RESEARCH

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Antimicrobial Resistance in *Streptococcus Pneumoniae* in Finland with Special Reference to Macrolides and Telithromycin

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Academic dissertation

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ACADEMIC DISSERTATION

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Summary

Aims and methods: This thesis investigated the prevalence of and trends in antimicrobial resistance in pneumococci in Finland, determined the genetic basis of macrolide resistance and evaluated the level of telithromycin non-susceptibility prior to its widespread usage. In addition, the clonality of telithromycin-resistant and penicillin-resistant isolates was examined. The study includes two sets of bacterial isolates: the first set consisted of 1007 non-invasive and invasive pneumococci collected in 2002 and the second set of isolates included all invasive pneumococci (n = 3571) collected in Finland in 2002-2006. Agar plate dilution in 5% CO_2, CLSI broth and disk diffusion methods were used for antimicrobial susceptibility testing. PCR was used to detect macrolide resistance genes and pyrosequencing to search for ribosomal mutations in domains V and II of 23S rRNA, and in genes encoding ribosomal proteins L4 and L22. The clonality of the bacteria was investigated with PFGE and multilocus sequence typing (MLST).

Results: Of the 1007 pneumococci collected in 2002, 21.5%, 12.1%, and 14.4% were non-susceptible to erythromycin, penicillin and tetracycline, respectively. Multiresistance was detected in 10.5% of the isolates. Only 0.1% of the isolates were non-susceptible to ceftriaxone (non-meningitis breakpoint) and <1.5% to fluoroquinolones. Two isolates were non-susceptible to linezolide. In 2002-2006, erythromycin resistance increased from 16% (2002) to 28% (2006) (Poisson regression, $p < 0.0001$), penicillin non-susceptibility from 8% to 16% (< 0.0001) and penicillin resistance from 0.8% to 3.7% ($p = 0.03$). Tetracycline resistance remained stable (~10%), as did the proportion of multiresistant isolates (~5%). Levofloxacin and ceftriaxone resistance was rare. Serotypes 14, 9V, 6B, 19F and 19A were the most frequently non-susceptible to erythromycin or penicillin. In both sets of collections of pneumococci, the highest prevalence of erythromycin resistance was among isolates derived from 0- to 2-year-old children: in 2006, 45.8% of isolates were resistant to erythromycin in this age group.

In 2002, disk diffusion testing revealed 26/1007 (2.6%) pneumococcal isolates that produced one to several clearly visible colonies inside the growth inhibition zone, indicating heterogeneous resistance to telithromycin. The telithromycin MIC_{50} and MIC_{90} of these isolates were 2 and 4 mg/L (range 0.063 to 8 mg/L), respectively, when measured by the agar dilution method, but with CLSI broth microdilution in a normal atmosphere the

MIC_{50} and MIC_{90} were 0.125 and 1 mg/L, respectively (range 0.063 – 2 mg/L). The telithromycin MIC_{50} and MIC_{90} of the zone isolates (isolated from the colony growing inside of the inhibition zone) was 32 mg/L and 64 mg/L, respectively, according to the agar dilution method in 5% CO_2, whilst they were 4 and 8 mg/L with CLSI broth microdilution in ambient air. This type of telithromycin resistance has not previously been described. All such isolates were *erm*(B) positive and two of them also carried *mef*(E), but the exact underlying mechanism of telithromycin resistance remained unresolved. Telithromycin resistant isolates had seven distinct sequence types, of which ST193 was the most frequent (n = 19). Other sequence types were 133, 273, 271, 2248, 2306 and 2307. PFGE results were in accordance with the MLST results. ST193 isolates were all 19A serotype variants of the PMEN clone Greece^{21}-30, while ST273 is a representative of the PMEN global clone Greece^{6B}-22 and ST271 is a single locus variant of a multi-drug-resistant Taiwanese^{19F} ST236 clone.

Among penicillin resistant isolates in 2002-2006, a total of 25 sequence types were found that distributed into ten clonal lineages (clonal complexes, CC). The most common clonal complex was CC156, accounting for 61% of all penicillin-resistant isolates, followed by CC271 (10% of the isolates) and CC81 (9%). The majority of the penicillin-resistant pneumococci in this study were representatives of single to triple locus variants of the following PMEN clones: Spain^{9V} ST156, Taiwan^{19F} ST236, Spain^{23F} ST81, and England^{14} ST9.

In 2002, the most frequent macrolide resistance gene was the *mef* gene (49%), followed by *erm*(B) (41%). A double mechanism [*mef*(E)+*erm*(B)] was detected in 5 (2.3%) isolates. Of the *mef* genes, 89% had the *mef*(E) subclass and 11% had *mef*(A). Mutation was detected in 16 isolates, of which 14 isolates (6.4%) had no other known resistance factor. Six new ribosomal protein mutations were recorded in this study. Of these, four mutations were in the L4 protein ($_{68}E_{69}$, $_{68}GQK_{69}$, $T_{94}I$, $V_{205}G$) and two in the L22 protein ($R_{22}C$, $A_{101}P$). In 2002-2006 the macrolide gene distribution was similar: the *mef* gene was detected in 56% of the investigated isolates (n = 223), while *erm*(B) was present in 31% and both *mef*(E) and *erm*(B) in two isolates (0.9%). Of the *mef*-positive isolates that were further investigated (n = 60), 72% had *mef*(E) and 28% *mef*(A).

Conclusions: The main observation of this thesis was the presence of heterogeneous telithromycin resistance among pneumococci carrying *erm*(B). Such isolates harbour a minor population of bacterial cells capable of expressing high level telithromycin resistance *in vitro*, which may be clinically significant. Because CLSI broth microdilution does not favour the detection of this resistance type, the disk diffusion susceptibility testing of *erm*(B)-positive pneumococci is recommended. Due to the high prevalence of resistance, macrolides cannot be recommended as the first line drugs for the treatment of pneumococcal infections. Apart from macrolide resistance, the proportion of penicillin non-susceptible isolates is rising.

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Tiivistelmä

Tavoitteet ja metodit: tässä tutkimuksessa selvitettiin pneumokokkibakteerin mikrobilääkeresistenssin esiintyvyyttä ja trendejä Suomessa ja testattiin kantojen herkkyyttä telitromysiinille ennen sen laajamittaista käyttöönottoa. Lisäksi tutkittiin makrolidiresistenssimekanismeja sekä resistenttien pneumokokkien klonaalisuutta. Tutkimus perustuu kahteen aineistoon, joista ensimmäinen käsittää 1007 pneumokokkikantaa, jotka on kerätty vuonna 2002. Toinen aineisto käsittää kaikki Suomessa eristetyt invasiiviset pneumokokit vuosilta 2002–2006 (n = 3571). Mikrobilääkeherkkyystestaus suoritettiin maljalaimennosmenetelmällä 5 % hiilidioksidiatmosfäärissä. Lisäksi käytettiin CLSI:n nestelaimennos- ja kiekkodiffuusiomenetelmiä. Makrolidiresistenssigeenejä etsittiin PCR-menetelmällä. Pyrosekvensointia ja sekvensointia käytettiin ribosomaalisten mutaatioiden osoittamiseen (domeeni V, 23S rRNA, ja ribosomaaliset proteiinit L4, L22). Bakteerien klonaalisuutta tutkittiin PFGE ja MLST – tekniikoilla.

Tulokset: Vuonna 2002 kerätystä 1007 pneumokokkikannasta 21.5 %, 12.1 %, 14.4 % ja 26.8 % oli resistenttejä tai herkkyydeltään heikentyneitä erytromysiinille, penisilliinille ja tetrasykliinille. Kannoista 1.5 % oli multiresistenttejä. Vain 0.1 % kannoista oli herkkydeltään heikentyneitä keftriaksonille (ei-meningiitti raja-arvo) ja <1.5 % fluorokinoloneille. Kahden kannan herkkyys linetsolidille oli heikentynyt. Vuosina 2002–2006, erytromysiiniresistenssi nousi 16 %:sta (2002) 28 %:iin (2006) (Poisson regressio, $p < 0.0001$). Penisilliinille ei-herkkien kantojen osuus nousi 8 %:sta 16 %:iin (< 0.0001) penisilliinille resistenttien kantojen osuus 0.8 %:sta 3.7 %:iin ($p = 0.03$). Tetrasykliiniresistenssi pysyi tasaisena (~10 %), kuten myös multiresistenttien kantojen osuus (~5 %). Levofloksasiini- ja keftriaksoniresistenssi oli harvinaista. Serotyypeistä 14, 9V, 6B, 19F and 19A olivat useimmin penisilliinille tai erytromysiinille resistenttejä. Molemmissa aineistoissa korkein makrolidiresistenssi havaittiin pneumokokeissa, jotka olivat eristetty 0-2 vuotiailta lapsilta. Vuonna 2006 tässä ikäryhmässä jo 46 % pneumokokeista oli resistentteijä erytromysiinille.

Vuonna 2002 kerätyistä pneumokokeista 26 (2.6 %) kannassa todettiin kiekkoherkkyystestauksessa heterogeeninen resistenssi telitromysiinille. Näillä kannoilla telitromysiinikiekon estovyöhykkeen sisällä havaittiin selkeitä pesäkkeitä, joiden lukumäärä vaihteli. Maljalaimennosmenetelmällä näiden kantojen telitromysiini MIC_{50} ja MIC_{90} arvot olivat vastaavasti 2 ja 4 mg/L (vaihteluväli 0.063-8 mg/L), mutta CLSI:n nestelaimennos-menetelmällä huoneilmassa 0.125 ja 1 mg/L (vaihteluväli 0.063 – 2 mg/L). Estovyöhykkeen sisältä eristetyissä ns. vyöhykekannoissa maljalaimennosmenetelmän telitromysiini MIC_{50} ja MIC_{90} arvot olivat 32 mg/L and 64 mg/L, mutta 4 ja 8 mg/L CLSI:n nestelaimennosmenetelmällä. Aiemmin tällaista

telitromysiiniresistenssiä ei ole dokumentoitu. Kaikilla telitromysiini-resistenteillä kannoilla oli *erm*(B) makrolidiresistenssigeeni ja lisäksi kahdella oli myös *mef*(E). Telitromysiiniresistenssin tarkempi mekanismi ei kuitenkaan tutkimuksissa selvinnyt. Telitromysiiniresistentit pneumokokit olivat seitsemää eri sekvenssityyppiä. Yleisin sekvenssityyppi oli ST193 (n = 19). Muita sekvenssityyppejä olivat 133, 273, 271, 2248, 2306 and 2307. PFGE tulokset vastasivat MLST tuloksia. ST193 kannat olivat kaikki serotyyppiä 19A ja ovat siten PMEN kloonin Greece^{21}-30 variantteja. ST273 edustaa maailmanlaajuisesti levinnyttä Greece^{6B}-22 kloonia, ja ST271 eroaa yhden lokuksen osalta multiresistentistä Taiwanese^{19F} ST236 – kloonista.

Vuonna 2002–2006 penisilliiniresistenttit kannat olivat 25 eri sekvenssityyppiä, jotka muodostivat 10 klonaalista linjaa. Yleisin linja oli CC156, johon kuului 61 % kaikista penisilliiniresistenteistä kannoista. Kymmenen prosenttia kannoista kuului klonaaliseen linjaan CC271 ja 9 % CC81 -linjaan. Valtaosa penisilliinille resistenteistä pneumokokeista oli yhden tai kahden lokuksen variantteja seuraavista PMEN klooneista: Spain^{9V} ST156, Taiwan^{19F} ST236, Spain^{23F} ST81, and England^{14} ST9.

Vuonna 2002 yleisin makrolidiresistenssimekanismi oli *mef* geeni (49 %). *erm*(B) geeni löytyi 41 % erytromysiinille resistenteistä pneumokokeista. Kaksoismekanismi [*mef*(E)+*erm*(B)] havaittiin 5 (2.3 %) kannassa. *mef* geeneistä yleisin oli *mef*(E) alatyyppi (89 %). Kuudellatoista makrolidiresistentillä pneumokokilla löytyi mutaatio joko ribosomissa (domeeni V 23S rRNA) tai ribosomaalisissa proteiineissa (L4, L22). Näistä 14 (6.4 %) kannalla ei ollut muuta tunnettua resistenssitekijää. Mutaatioista kuusi oli ennen julkaisemattomia. Näistä 4 oli L4 proteiinissa ($_{68}E_{69}$, $_{68}GQK_{69}$, $T_{94}I$, $V_{205}G$) ja kaksi L22 proteiinissa ($R_{22}C$, $A_{101}P$). Vuosina 2002–2006 invasiivisten pneumokokkien makrolidiresistenssigeenijakauma oli samankaltainen: *mef* geeni todettiin 56 % tutkituista kannoista ja *erm*(B) 31 % kannoista. Kaksoismekanismi todettiin kahdella kannalla (0.9 %). *mef*-positiivisista kannoista tutkittiin tarkemmin 60 kantaa, näistä 72 % oli *mef*(E) alatyyppiä ja 28 % oli *mef*(A) alatyyppiä

Johtopäätökset: Tämän tutkimuksen päähavainto oli pneumokokkien heterogeenisen telithromysiiniresistenssin löytyminen. Yhteistä kannoille oli *erm*(B) makrolidiresistenssigeenin löytyminen genomista. Tällaisilla bakteerikannoilla on yksittäisiä bakteerisoluja, jotka ovat kykeneviä ilmentämään korkea-asteista telitromysiiniresistenssiä laboratoriolosuhteissa. Korkeiden MIC arvojen perusteella kuvatunkaltaisella resistenssillä saattaa olla kliinistä merkitystä. Koska CLSI:n nestelaimennosmenetelmä ei suosi heterogeenisen telitromysiiniresistenssin havaitsemista, *erm*(B)-positiiviset tai erytromysiinille muuten korkeasti resistentit pneumokokit tulisi testata kiekkoherkkyysmenetelmällä. Tutkimuksessa havaittiin myös että pneumokokkien makrolidiresistenssi on huolestuttavasti lisääntynyt ja että se on erityisen korkea kannoilla, jotka ovat peräisin pienten lasten infektioista. Tästä syystä makrolideja ei voi suositella ensisijaislääkityksenä pneumokokki-infektioiden hoitoon. Makrolidiresistenssin ohella myös penisilliinille herkkyydeltään heikentyneiden kantojen osuus nousi jyrkästi tutkimusajanjaksolla.

Contents

Abbreviations

A2059	adenine in the position of 2059 of domain V of the 23S rRNA (*Escherichia coli* numbering)
A2059G	adenine replacement by guanine at position 2059
AOM	acute otitis media
ATP	adenosine triphosphate
AUC	area under the concentration curve
CLSI	Clinical Laboratory Standards Institute
CSF	cerebrospinal fluid
erm	erythromycin ribosomal methylation gene
ERY	erythromycin
gyr	gyrase gene
I	intermediate
IPD	invasive pneumococcal disease
M	macrolide resistance phenotype (resistance to 14- and 15-membered macrolides)
mef	macrolide efflux gene
mega	macrolide efflux genetic assembly
MIC	minimum inhibitory concentration
ML	macrolide-lincosamide resistance phenotype
$MLKS_B$	macrolides, lincosamides, ketolides and streptograminB
MLS_B	macrolide-lincosamide-streptogramin B resistance phenotype
MLST	multilocus sequence typing
msr	macrolide streptogramin resistance gene
par	topoisomerase gene
PBP	penicillin binding protein
PCR	polymerase chain reaction
PEN	penicillin
PFGE	pulsed field gel electrophoresis

PNSP	penicillin non-susceptible (I+R isolates)
PCV-7	heptavalent pneumococcal conjugate vaccine
R	resistant
rRNA	ribosomal ribonucleic acid
S	susceptible
ST	sequence type
SXT	trimethoprim-sulfamethoxazole
TEL	telithromycin
tet	tetracycline resistance gene
TET	tetracycline
Ala, A	alanine
Arg, R	arginine
Asn, N	asparagine
Asp, D	aspartic acid
Cys, C	cysteine
Glu, E	glutamic acid
Gln, Q	glutamine
Gly, G	glycine
His, H	histidine
Ile, I	isoleucine
Leu, L	leucine
Lys, K	lysine
Met, M	methionine
Phe, F	phenylalanine
Pro, P	proline
Ser, S	serine
Thr, T	threonine
Trp, W	tryptophan
Tyr, Y	tyrosine
Val, V	valine

List of original publications

This thesis is based on the following four original papers, referred to by their Roman numerals.

I Rantala M, Huikko S, Huovinen P, Jalava J. Prevalence and molecular genetics of macrolide resistance among Streptococcus pneumoniae isolates collected in Finland in 2002. Antimicrob Agents Chemother. 2005 Oct;49(10):4180-4.

II Rantala M, Haanperä-Heikkinen M, Lindgren M, Seppälä H, Huovinen P, Jalava J. Streptococcus pneumoniae isolates resistant to telithromycin. Antimicrob Agents Chemother. 2006 May;50(5):1855-8.

III Rantala M, Nyberg S, Lindgren M, Huovinen P, Jalava J, Skyttä R, Teirilä L, Vainio A, Virolainen-Julkunen A, Kaijalainen T. Molecular epidemiology of telithromycin-resistant pneumococci in Finland. Antimicrob Agents Chemother. 2007 May;51(5):1885-7.

IV Siira L, Rantala M, Jalava J, Hakanen A , Huovinen P, Kaijalainen T, Lyytikäinen O, Virolainen A. Temporal trends of antimicrobial resistance and clonality of invasive *Streptococcus pneumoniae* isolates in Finland, 2002-2006. In press.

1. Review of the literature

1.1 Introduction

Streptococcus pneumoniae or pneumococcus belongs to the genus *Streptococcus*, family *Streptoccocceae*. Pneumococcus was first microscopically observed in 1875 by Klebs in samples from the lungs of pneumonia patients. Two researchers, Sternberg in the USA and Pasteur in France, independently demonstrated the pathogenicity of the organism in 1881 by inoculating rabbits with saliva containing pneumococcus. Pasteur was the first to successfully cultivate the organism from infected rabbits (19). Today, pneumococcus is known as one of the major human pathogens worldwide, causing a wide variety of infections. These infections can be treated with antimicrobials, but emerging antimicrobial resistance in *S. pneumoniae* is one of the major public health concerns.

Characteristics of *S. pneumoniae*

S. pneumoniae is a Gram-positive coccus that is catalase-negative, non-motile, non-sporing and produces typical greenish haemolysis (alphahaemolysis) in a blood agar base. It requires sera to grow and tends to grow in pairs or short chains (212). Pneumococci can be differentiated from other alphahaemolytic or viridians streptococci by their susceptibility to optochin, bile solubility and capsular reaction to diagnostic pneumococcal sera (serotyping) (162). Primary identification of pneumococcus in clinical laboratories is frequently based on the typical colony morphology and susceptibility to optochin, although some atypical pneumococcal strains can show optochin resistance (3, 213), thus making identification sometimes difficult. Apart from serotyping, pneumococci can be further identified, for instance, by commercial kits such as Api Rapid Strept, Rapid ID 32 and VITEK (bioMerieux, Marcy l'Etoile, France), DNA probe analysis (AccuProbe, GEN-PROBE, San-Diego, Ca), PCR (252), sequencing or MLST (149).

The pneumococcus has many virulence factors, of which the polysaccharide capsule is one of the most important (185). The capsule protects the bacterial cell from phagocytosis and inhibits complement activation (212). Capsular polysaccharides are antigenic and they induce a specific antibody response in

the host. So far, 91 different capsular serotypes have been detected (161, 267). Pneumococcus is also capable of switching from one capsule type to another (serotype switch) (45, 133, 186). Non-capsulated forms of pneumococci also exist (26). Other virulence factors include pneumolysin toxin, which is able to lyse eucaryotic cells, pneumococcal surface protein A, which has a role in protecting bacteria from attack by host immune defence mechanisms (184, 185), and pneumococcal pilus, a hair-like protein extending from the surface of the bacterial cell, which enhances the adhesion of bacteria to respiratory epithelial cells and stimulates the host immune response increasing the pathogenicity of an isolate (22, 156). Additional factors that may play a role in virulence include autolysins, signal peptidases and numerous surface proteins, but their role is not yet clearly defined (184).

Epidemiology of pneumococci

S. pneumoniae is a common inhabitant of the normal nasal microbiota of humans. Pneumococci can be isolated from 2 to 65% of healthy people (36, 129, 160, 217, 226, 259, 301, 328, 336), the carrier rate being higher in children than in adults (139, 160). Carriers can harbour different pneumococcal strains at the same time (14). Pneumococcus is the major pathogen in acute otitis media (AOM) and community-acquired pneumonia and a frequent causative organism in invasive bacterial infections such as septicaemia and meningitis. In addition, pneumococcus can cause sinusitis, cellulitis, endocarditis, fasciitis, abscesses, peritonitis, septic arthritis, and pelvic infections (212). Factors associated with an increased risk for invasive pneumococcal infection include age (< 2 and > 65 years), male sex, smoking, alcoholism, institutionalization, day-care attendance, immune deficiencies, and other co-morbidities such as chronic obstructive pulmonary disease, diabetes mellitus and cardiovascular disease (269).

Pneumococcal diseases cause significant morbidity and mortality, leading to a high burden and costs to health care systems worldwide. In developed countries, mortality from invasive pneumococcal infections ranges from <1 to 30% depending on age and underlying condition (16, 118, 190, 269), but can be up to 50% in developing countries (37). In Finland, 500 000 cases of AOM have been estimated to occur each year (254), of which pneumococcus is an aetiological factor in 26%-60% of the cases (207). Annual incidence estimates for *S. pneumoniae* related community-acquired pneumonia for children and the elderly > 60 years of age in Finland are 6.4/1000 (159) and 8/1000 (197), respectively. According to the National Infectious Disease Register, more than 700 cases of invasive pneumococcal disease (IPD) are reported annually in Finland, giving an incidence of 14 cases / 100 000

inhabitants, the disease burden being the highest in 0- to 4–year-olds (38/100 000) and those aged 65 years and older (28/100 000) (http://www3.ktl.fi). The incidence has remained relatively stable within the past few years.

Vaccines for preventing pneumococcal infection

Two types of vaccine against pneumococcal infection are in clinical use: a capsular polysaccharide and a conjugate vaccine (277). The pneumococcal polysaccharide vaccine (Pneumovax, Sanofi Pasteur, Belgium) contains the 23 most common capsular polysaccharide antigens: 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F, and induces a B-cell response in the host. The vaccine's effectiveness in preventing IPD is 48-81% in adults with normal immune systems (277). It does not reduce the incidence of community-acquired pneumonia, but decreases the disease severity, the risk for bacteremia and mortality (277). In particular, this vaccine has been shown effective in preventing the consequences of pneumonia and mortality among >65 year olds if given together with the influenza vaccine (63). The target groups for this vaccine are ≥ 65–year-olds and those ≥ 5-year-olds who are at risk of acquiring severe pneumococcal infection (277).

The polysaccharide vaccine does not induce protective immunity in children under five years (93). Therefore, a heptavalent conjugate vaccine (PCV-7) has been developed (Prevenar; Wyeth Lederle Vaccines S.A., Belgium). It contains polysaccharides from seven different capsule types known to cause the majority of invasive pneumococcal disease in children: 4, 6B, 9V, 14, 18C, 19F and 23F. These are linked to a highly immunogenic carrier protein (372). The PCV-7 vaccine induces both B and T cell responses as well as mucosal immunity. PCV-7 has been proven safe and immunogenic in infants and toddlers (313) and very effective in reducing the incidence of IPD and other pneumococcal disease in children, as well as in other age groups (279, 355). Early clinical trials reported a vaccine efficacy of 100-97% against IPD caused by vaccine serotypes in vaccine target groups (33). Later, it was illustrated that after introducing the vaccine in the USA, IPD caused by vaccine serotypes decreased 94% from 80 / 100 000 in 1998-1999 to 4.6 / 100 000 in 2003 in children < 5 years, and that overall the reduction in IPD incidence in this age group was 75%, from 96.7 to 23.9 / 100 000 (55). In addition, the total incidence of IPD decreased by 29% in the whole population. The reduction occurred in all age groups, but was most prominent in the elderly (55). Early studies also showed that in addition to reducing the incidence of IPD, PCV-7 vaccinations reduced AOM cases or the rate of visits to physicians due to AOM by 6-9% in infants (104, 127), while larger

scale studies showed a 20-40% decline in AOM health care visits on the population level (140, 371). Although the results of PCV-7 in reducing the burden of pneumococcal diseases have so far been promising, the replacement of vaccine serotypes by serotypes not covered by the vaccine possesses a threat to this development (88, 314).

1.2 Antimicrobials used for treating pneumococcal infections

Macrolides, lincosamides and streptogramines

Erythromycin was the first macrolide introduced into clinical use in the early 1950s. Erythromycin is an organic compound produced by the actinomycete *Streptomyces erythraeus*, currently known as *Saccharopolyspora erythraea* (368). Apart from *S. pneumoniae*, erythromycin is active against Gram-positive cocci, mycoplasma, *Chlamydia*, *Campylobacter*, *Bordetella*, *Moraxella, Neisseria* and spirochaete species, as well as many anaerobic bacteria. Due to the short half life and poor acid stability of erythromycin, research was carried out on 14-, 15- and 16-membered macrolides that led to the discovery of new macrolide compounds in the late 1980s and 1990s. The newer macrolides have better acid stability and more favourable pharmacokinetic profiles (48). They also have improved antimicrobial activity against mycoplasma and many Gram-negative species (48, 153).

The basic structure of macrolides is a large lactone ring. Erythromycin A has a 14-membered lactone ring to which L-cladinose and an amino sugar, D-desoamine, are attached. Other 14-membered macrolides include roxithromycin, dirithromycin and clarithromycin, which are derivatives of erythromycin (48). Azithromycin is a 15-membered semisynthetic derivative of erythromycin belonging to the azalides because it has methylated nitrogen inserted in a lactone ring (Figure 1) (21). Spiramycin, rokitamycin, tylosin, josamycin, mideacamycin and miocamycin have a 16-membered lactone ring (218).

Macrolides have binding sites in the bacterial ribosome. Erythromycin binds to domain V of the 23S rRNA within a tunnel of the peptidyltransferase centre, which serves as a channel for a growing peptide chain. The surface of the tunnel is formed by domains V and I of 23S rRNA, and by ribosomal proteins L22 and L4 (281). The key positions for erythromycin binding are A2058 and A2059 (*Escherichia coli* numbering), A2505, A2062, and U2609 in domain V. Because hairpin 35 of domain II is also in the vicinity of this binding site, A752 may have a role in the binding of the erythromycin. Erythromycin blocks the polypeptide exit tunnel and thus prevents the

extension of the growing peptide and provokes the premature release of the unmature peptide chain. It also prevents ribosomal assembly at an early stage of protein synthesis (218).

Lincomycin and clindamycin, a semi-synthetic derivative of the former, belong to the lincosamides. Although structurally very different from macrolides, lincosamides share a similar mechanism of action with them, as also do streptogramins (99). Streptogramins are comprised of two components, streptogramin A (dalfopristin, pristinamycin II, or virginiamycin M) and B (quinupristin, pristinamycin I, or virginiamycin S). Alone, these components have weak bacteriostatic activity, but their mixture is bactericidal and synergistic. Attachment of type A components to a bacterial ribosome leads to a conformational change within the peptidyl transferase centre that increases type B component affinity 100-fold (154). Streptogramin A blocks the substrate sites of the peptidyltransferase centre. Type A streptogramins prevent the earliest event of peptide chain elongation, whereas type B streptogramins interfere with the formation of the growing polypeptide chain similarly to macrolides (339). Figure 1 illustrates the structures of some macrolides, clindamycin, quinupristine and telithromycin.

Ketolides

Ketolide antimicrobials are a relatively new, recently developed antimicrobial group, of which telithromycin (HMR-3647 or RU-66647) and cethromycin (ABT-773) are examples (47, 147). Telithromycin was the first ketolide introduced into clinical practice at the beginning of the 21st century (363). Its antimicrobial spectrum is similar to that of newer macrolides, although it is more active (lower MICs) against many bacterial species. Telithromycin is a semi-synthetic derivative of erythromycin A composed of a 14-membered lactone ring in which the neutral L-cladinose sugar has been replaced by a keto group at position C3 (Figure 1). Telithromycin also has a C11/12 alkyl-aryl extension linked to a carbamate (47). Telithromycin inhibits protein synthesis by interacting with domains II and V of 23S rRNA of assembled ribosomes, and with part-assembled 50S precursors, causing nucleolytic degradation of the precursor particles (1, 47). It has binding sites similar to macrolides, but in addition has a binding site at position A752 in hairpin 35 of domain II (94). Simultaneous interaction both with domain V and domain II strengthens binding of the drug to resistant ribosomes, making telithromycin a potent drug against macrolide-resistant pneumococci (94). Telithromycin concentrates inside neutrophils and macrophages, the drug concentration being several times higher in intracellular compared to extracellular fluid (270). Telithromycin has inoculum-dependent

bacteriostatic and concentration-dependent activity against most respiratory pathogens. In addition, a significant post-antibiotic effect, which is concentration dependent, has been observed (179, 248), although it is shorter against erythromycin-resistant than susceptible pneumococci (248). Telithromycin's main indication is the treatment of community-acquired pneumonia. In clinical trials the efficacy of telithromycin has been reported to be better than or comparable to other macrolides or betalactams (144).

Penicillins and cephalosporins

The core structure of penicillins and other betalactams is a beta-lactam nucleus. Beta-lactam antimicrobials inhibit the peptideglycan synthesis of the bacterial cell wall by binding irreversively to the active site of penicillin-binding proteins (PBPs) leading to osmotic hydrolysis of the bacterial cell (351).

Betalactam drugs are widely used to treat streptococcal infections. In many countries, including Finland, aminopenicillins or penicillin are the first line drugs for treating acute otitis media and community-acquired pneumoniae (9, 17, 18, 125, 157, 256, 272, 285, 286). Cephachlor and cefuroxime are examples of second generation cephalosporins, which are recommended as second or third line drugs for such infections (71) Ceftriaxone is a third generation cephalosporin used for treating severe life-threatening pneumococcal infections such as meningitis (178). Cephalosporins share a similar mechanism of action with penicillins, but they have a wider antimicrobial spectrum (142). First generation cephalosporins are mainly active against Gram-positive organisms such as streptococci and staphylococci, whilst the spectrum of the later generation cephalosporins is more focused on Gram-negative bacteria (23, 142, 284).

Other antimicrobials

Fluoroquinolones are synthetic antimicrobials that prevent bacterial DNA synthesis by inhibiting the action of DNA gyrase (359). Fluoroquinolones are bactericidal and have a wide spectrum of activity. Compared to ciprofloxacin, newer fluoroquinolones such as moxifloxacin, levofloxacin and gatifloxacin are more active against Gram-positive cocci, including pneumococcus (257, 304, 311, 337), although the results of one meta-analysis pointed to a similar efficacy in clinical situations (241). It has been suggested, however, that the use of older fluoroquinolones such as ciprofloxacin should be avoided in treating pneumococcal infections because it can favour the selection of clones resistant to newer fluoroquinolones (196).

Tetracyclines were discovered in the late 1940s. Tetracyclines have wide antimicrobial spectrum covering gram-positive and gram-negative bacteria, chlamydiae, mycoplasmas, rickettsiae, and protozoan parasites. Tetracycline molecule is composed of four linearly fused six-membered hydrocarbon rings. Examples of this class antimicrobials are chlortetracycline, oxytetracycline, doxycycline and recently developed tigecycline. They inhibit protein synthesis in the bacterial cell by binding to the 16S rRNA part of the 30S subunit of the bacterial ribosome (242).

Sulfonamides were first time used in the early 1930s while the use of trimethoprim started in the late 1960s. Soon trimethoprim-sulfonamide combination became common because it was considered to act synergestically. Both compounds have a wide antimicrobial spectrum covering many respiratory pathogens including pneumococcus as well as other bacteria, such as *Staphylococcus aureus* and members of the family *Enterobactericeae*. Trimethoprim-sulfonamides prevent sequential steps in bacterial folic acid synthesis (173)

Vancomycin is a tricyclic glycopeptide antimicrobial produced by *Amycolatopsis orientalis* a nocardioform actinomycete. Vancomycin acts by inhibiting the synthesis of the cell wall of gram-positive bacteria. Linezolide belongs to oxazolidinone class of antimicrobials that are inhibitors of protein synthesis. However, unlike many other protein synthesis inhibitors, linezolide acts at the initiation phase of protein synthesis by preventing 30S and 50S subunits of the ribosome from binding to each other. Linezolide and vancomycin are used to treat severe infections caused by Gram-positive antimicrobial resistant organisms, including pneumococci. They are often considered as reserve drugs for treating life threating infections (57, 347).

1.3 Antimicrobial resistance mechanisms

MLKSB antimicrobials

Macrolide resistance is mediated by two main mechanisms in pneumococcus: by target site modification and active drug efflux (56). The most important form of target site modification in pneumococci is methylation of ribosomal adenine base A2058 by methylases (rRNA adenine N^6 methyltransferase), leading to a reduced affinity of macrolides to ribosomes. Target site

modification can also be achieved via ribosomal mutations. Active drug efflux is mediated via efflux pumps and is the prevailing resistance mechanism, along with ribosomal methylation in pneumococci (107). Enzymes that inactivate macrolides or lincosamides have not been described in this bacterial species.

Methylases

Ribosomal modification by methylation as a mechanism for macrolide resistance was first described in the early 1970s (352). Dozens of different types of methylase genes have been detected and sequenced in several species such as *Streptococci, Staphylococci*, *E. coli*, *Enterococci, Clostridium perfringens*, *Lactobacillus reuteri*, *Arthrobacter luteus*, *Corynebacterium difteriae*, *Bacteroides fragilis*, *Bacillus* and *Streptomyces*. Methylase enzymes catalyse either mono- or dimethylation of a particular adenine residue in the 23 rRNA (352). In *S. pneumoniae* the prevailing methylase gene is *erm*(B), which was originally designated as *erm*(AM) and was initially found from a plasmid pAM77 of *Streptococcus sanguis* (218, 352). The ErmB enzyme predominantly catalyses the dimethylation of the ribosomal adenine base at position 2058 of domain V in 23S rRNA, leading to a reduced affinity of erythromycin for ribosomes. Dimethylation of this site confers cross resistance to 14-, 15- and 16-membered macrolides as well as to clindamycin and streptogramin B. Consequently, this type of resistance is termed MLS_B resistance (353). Due to the synergistic effect of streptogramin A and B, a combination of streptogramins is effective against isolates showing the MLS_B phenotype, although MICs may be slightly elevated (346). The other methylase gene in pneumococcus, although rarely present, is *erm*(A), which was originally designated as *erm*(TR) (309). *erm*(TR) was first described by Helena Seppälä and her co-workers, who observed it in *Streptococcus pyogenes* (309). Because *erm*(TR) is closely related to *erm*(A) of *Staphylococcus aureus*, it was later recommended that the name *erm*(A) should be used instead of *erm*(TR) so as to avoid complexity in the nomenclature (294). *erm*(A) and *erm*(B) share only 58% similarity at the nucleotide level (309).

Resistance to MLS_B antimicrobials may be constitutive or inducible in isolates harbouring the *erm* gene (353). Phenotypes of inducible strains show resistance to 14-, 15- and 16-membered macrolides, but susceptibility to clindamycin and/or streptogramin B is variable (56) After the incubation of such isolates in a low concentration of 14- or 15-membered macrolides, an elevation of MICs of clindamycin and streptogramin-B can be observed (353). In disk diffusion susceptibility testing, the induction is manifested by

D-shape blunting of the growth inhibition zone around the lincosamide or streptogramin disk adjacent to the 14- or 15-membered macrolide disk (353). The inducibility of the *erm*(B) gene is related to the leader sequence the preceding the methylase gene. Mutations or deletions in the leader peptide can convert inducible resistance to the constitutive form (353). Bacterial phenotypes with constitutive MLS_B resistance are highly resistant to these antimicrobials (218).

Pneumococcal *erm* genes locate in numerous transposons, which spread either by transformation or conjugation. Transposons have inverted repeat (IRs) sequences at each end and carry genetic codes for transposases, enzymes that allow transposons to be cut from DNA and inserted at different positions in the genome. Insertion sequences (IS) are the simplest forms of transposons. Composite transposons contain the insertion elements at either end, but can contain other genes in the middle. These types of transposons are usually very large because they can contain derivatives of several smaller transposons. All *erm*(B)-carrying elements are derivatives of the tetracycline *tet*(M)-carrying Tn*916* transposon, which was originally detected in *Enterococcus faecalis* (128). Tetracycline determinants carried in the same transposons together with *erm*(B) can be silent (67). An example of a composite transposon in *S. pneumoniae* is Tn*3872*, in which *erm*(B) carrying transposon Tn*917* is integrated into Tn*916* (232). Other *erm*(B)-containing transposons in pneumococci include Tn*1545*, Tn*6003*, Tn*6002* (67). Tn1545 was the first transposon described in pneumococcus. It is a conjugative transposon containing *erm*(B), *tet*(M) and *aph*A-3 (kanamycin resistance) resistance genes with a size of 25.3 kb (72, 73). Tn*6002* (size 20.9 kb) evolved from the insertion of an *erm*(B)-containing DNA strand into Tn*916* (68). Tn*6003* is a 25.1 kb composite transposon carrying the same resistace genes as Tn*1545* (68), but besides the kanamysin resistance determinant an additional *erm*(B) gene without a stop codon can exist (68). In pneumococci carrying *erm*(B) and *mef*(E), a *mef*-containing mega element is inserted in a transposon similar to Tn*2009*, forming a new 226.3 kb composite transposon Tn*2010* (85).

Active efflux of the drug

Until 1993, before Helena Seppälä and colleagues described a novel M-phenotype in *Streptococcus pyogenes* (308), it was thought that macrolide resistance in streptococci was exclusively mediated by *erm*(B) (325). Isolates of the M phenotype were observed to be resistant to 14- and 15-membered macrolides, but not to 16-membered macrolides, lincosamides or streptogramin B (308). Later, this phenotype was also described in *S.*

pneumoniae (325). In 1996 it was discovered that resistance in M phenotype pneumococci and streptococci was due to active drug efflux, since erythromycin uptake by the bacterial cell was increased in the presence of carbonylcyanide m-chlorophenylhydrazone (CCCP) or arsenate, the agents that disrupt proton motive force in strains with the M-phenotype (327). Finally, molecular cloning and functional analysis proved that the gene responsible for coding the efflux pump mechanism in *Streptococcus pyogenes* was *mef*(A) (GenBank accession number U70055) (64). Soon, Tait-Kamradt and co-workers (1997) described the presence of a similar gene, *mef*(E) (GenBank accession number U83667), in *S. pneumoniae* (331). *mef* genes are homologous to transporters using proton motive force, unlike msrA and msrB in staphylococci. Effux pumps coded by *mef* genes belong to the major facilitator superfamily (MFS), in which the extrusion of a drug is coupled with ion exchange (325). Both subtypes of *mef* genes, *mef*(A) and *mef*(E), have been detected in pneumococci (77, 86, 303). Sequencing analysis has revealed that these two genes are closely related, sharing 90% identity at the DNA level and 88% similarity at the protein level (331). Consequently, it was first suggested that they should be reported as a single gene, *mef*(A), to avoid conflicting interpretations and complexity in nomenclature (294). However, regardless of the high degree of identity between *mef*(A) and *mef*(E), numerous differences were later discovered. *mef*(A) and *mef*(E) were found to be carried by different genetic elements (77, 86). *mef*(A) of pneumococcus is part of a chromosomal element, a defective transposon designated to *Tn*1207.1 (303), while *mef*(E) is carried by a chromosomal insertion element, designated the macrolide efflux genetic assembly or mega (86). The mega element has at least five insertion sites in the pneumococcal genome (132). Erythromycin MICs of isolates which carry the *mef*(A) element were shown to be higher compared to isolates carrying *mef*(E) (8). Penicillin non-susceptibility is commonly found together with *mef*(E), but is not as frequent in the presence of *mef*(A) (15, 69, 86). Moreover, *mef(*A)-carrying isolates are usually clonally related, whilst *mef*(E) isolates have a more heterogenetic pattern (15, 69, 86). Because of these differences, it was suggested that the genes should be discriminated (86). Recently, one new variant of *mef* gene, designated as *mef*(I), was described in two pneumococcal isolates by Cochetti and co-workers (69). The new variant was not carried by a mega element. The amino acid sequence coded by *mef*(I) showed 96.5% similarity with that of *mef*(E) and 94.3% with the amino acid sequence coded by *mef*(A) (69). Later, it was observed that *mef*(I) is carried by a novel composite genetic element, designated as the 5216IQ complex. The size of this element is around 30 kb and it is composed of parts of the transposons Tn5252 and Tn916 and a new element designated as IQ (240).

An additional efflux mechanism, designated as *msr(*D) (8, 47) or *mel* (21) [hereafter *msr*(D)], has been found in all three *mef* carrying genetic elements in pneumococcus (77, 240). *msr*(D) is a homologue of the *msr*(A) determinant found in staphylococci (77), which codes an ATP-binding cassette (ABC) transporter that utilizes the energy derived from ATP hydrolysis to efflux drugs (325). *mef* and *msr*(D) genes are co-transcribed in pneumococci. The *msr*(D) gene has also been shown to be capable of conferring resistance to 14- and 15-membered macrolides without the *mef* determinant in pneumococcus (77). The expression of the *mef-msr*(D) efflux mechanism has been illustrated to be inducible by a low concentration of 14- and 15-membered macrolides, elevating their MICs, but does not affect the MICs of 16-membered macrolides, clindamycin or streptogramin B (6).

Ribosomal mutations

Macrolide-resistant pneumococci that do not harbour common resistance genes usually have ribosomal mutations that appear to cluster in the peptidyltransferase region in domains V and II of 23S rRNA, or in 50S ribosomal protein coding genes L4 or L22. Mutations in these areas prevent the antimicrobial binding to its target site (218, 352). Phenotypes of mutated strains are variable, depending on the location of the mutation(s), the number of mutated alleles and probably the level of expression of the gene (108, 332, 333). Azithromycin is considered to be one of the most potent macrolides for selecting mutants (52). Laboratory experiments show that after serial passage of pneumococcal strains in azithromycin, mutations can be observed at positions A2058G, A2059G, C2611A and C2611G of the peptidyl transferase region at domain V of 23S rRNA. In addition, amino acid changes were detected in a highly conserved $_{63}$KPWRQKGTGRAR$_{74}$ region (333). These mutations have also been observed in clinical isolates. The most frequently reported ribosomal mutation in clinical isolates seems to be A2059G (92, 108, 290), while A2058G mutation is less frequent, although it is rather common in *Streptococcus pyogenes* (40, 116, 183). Table 1 summarises the different types of ribosomal mutations associated with macrolide resistance in clinical and laboratory pneumococcal strains.

Mechanisms of telithromycin resistance

Telithromycin has been reported to be active against erythromycin-resistant strains of *S. pneumoniae*, regardless of the resistance mechanism (182, 214, 215, 245, 354). Pneumococcal strains that harbour *erm* or *mef* genes have higher MICs for telithromycin than wild type isolates (0.5 vs. 0.015 mg/L), but their telithromycin MIC does not usually exceed the susceptibility

breakpoints set by CLSI (113, 182, 215, 245, 354). However, telithromycin resistance has been described in isolates in which there are mutations in the *erm*(B) leader sequence (166). In some cases, the telithromycin resistance mechanism is unclear but is somehow associated with the presence of *erm*(B) (349). In *mef*-carrying isolates, telithromycin MIC elevation may be linked to the *msr*(D) determinant instead of *mef*, since in laboratory experiments *msr*(D) transformants were observed to have higher telithromycin MICs than transformants with only the *mef* gene (77). Telithromycin has also been shown to be active against many pneumococcal strains that have a ribosomal mutation (108). However, there are some exceptions. In one report a *S. pneumoniae* isolate with an 18-base-pair insertion in the gene coding the L4 protein had a telithromycin MIC of 3.12 mg/L (332). Pihlajamaki and co-workers described a 12 base pair amino-acid insertion (Val-Arg-Pro-Arg) after position 277 in the gene encoding L22. The telithromycin MIC of this strain was 2 mg/L, but MICs to macrolides were relatively low (273). Mutations at the position of A752 in hairpin 35 of domain II have also been associated with telithromycin MIC elevation (166).

Other antimicrobials

Resistance against betalactams in pneumococci is mediated via changes in the genes encoding penicillin-binding proteins PBP1a, PBP2x, and PBP2b (138) and cell wall muropeptide branching protein MurM (126), leading to a reduced affinity of PBPs for the betalactam drugs. High level resistance is usually acquired by multiple mutations in the genes encoding PBPs. These genes are also called mosaic genes, referring to the long adjoining nucleotide sequences within PBP genes (59, 155). The acquisition of mosaic genes may occur via transformation from the same or closely related bacterial species (70, 146, 155). Pneumococcal isolates with a reduced susceptibility or resistance to penicillin often also have a diminished susceptibility to other betalactam antimicrobials, including newer cephalosporins (38, 122), although not necessarily to the extent that they would exceed non-susceptibility breakpoints. However, pneumococcal isolates with full resistance to penicillin are often also non-susceptible to second or third generation cephalosporins (122).

Resistance to fluoroquinolones is encoded by mutations in either *par*C or *par*E genes of topoisomerase IV or in *gyr*A or *gyr*B genes of DNA gyrase. These mutations can occur in combination or separately (2, 152, 263, 264, 276, 278, 370). They commonly appear in a stepwise fashion, leading first to a slightly decreased susceptibility to fluoroquinolones. Additional mutation in the other target gene leads to full resistance. Enhanced efflux of certain

fluoroquinolones, mediated by membrane-associated protein PmrA, has also been documented as a fluoroquinolone resistance mechanism in pneumococcus (42). It has been suggested that apart from spontaneous mutations, the horisontal transfer of genetic material might play role in the developement of fluoroquinolone resistance (175).

Tetracycline resistance in pneumococci is mediated via the *tet*(M) or *tet*(O) genes, which encode ribosomal protection proteins leading to a displacement of tetracycline from its binding site (357). Tetracycline resistance is frequently linked with erythromycin resistance because tetracycline determinants are carried by the same transposons as *erm(*B) (49, 67, 310). Therefore, high tetracycline resistance rates are usually reported by the countries in which high macrolide resistance percentages, due to *erm*(B), are observed.

Resistance to trimethoprim-sulfonamides is due to mutations in dihydrofolate reductase and dihydropteroate synthase, enzymes responsible for folic acid synthesis (173, 356) while point mutations in the genes coding 23S rRNA, such as G2576T, has been reported to mediate resistance to linezolid (235).

1.4 Occurrence of antimicrobial resistance

Susceptibility testing and breakpoints

Several methods have been developed for testing antimicrobial susceptibility in vitro. Roughly, these can be divided into dilution susceptibility tests and disk diffusion tests. The former tests measure the minimum inhibitory concentration (MIC) of an antimicrobial in mg/L or µg/ml that prevents bacterial growth, while the latter tests provide qualitative results usually classifying bacteria into three susceptibility categories: resistant (R), intermediate (I) or susceptible (S) (212). To interpret susceptibility testing results, breakpoints for the separate susceptibility categories need to be determined. The breakpoints should be based on the distribution of the (wild type) bacterial population, pharmacokinetic and dynamic parameters of the tested drug in question as well as clinical trials. Many existing breakpoints have been set in relation to achievable drug serum concentrations, but recently the importance of pharmacodynamic parameters in setting up breakpoints has also been addressed (7).

Although the primary aim of antimicrobial susceptibility testing is to provide information for a clinician to choose the optimal antimicrobial treatment for a

patient, its results can be used to follow changes in the antimicrobial resistance of certain bacterial populations over time (75). Many organizations, such as the British Society for Antimicrobial Chemotherapy, the Swedish reference group for antimicrobials, the Clinical Laboratory Standards Institute (CLSI, formerly NCCLS) in the USA and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) in the EU have worked to standardise and harmonise antimicrobial susceptibility testing and breakpoints (198). Due to the parallel work of many organisations, there is considerable variation in the breakpoints that have been set, making comparison of the data from different sources and studies challenging, although CLSI breakpoints have so far been widely used in the literature. The European breakpoints usually tend to be more conservative compared to CLSI breakpoints. For example, telithromycin breakpoints for the susceptible and resistant categories are, respectively, ≤ 1 and ≥ 4 mg/L according to the CLSI (66) and ≤ 0.25 and ≥ 1 mg/L according to EUCAST (http://www.srga.org/eucastwt/MICTAB/MICmacrolides.html). In addition, re-setting of breakpoints may affect greatly to susceptibility categorisation of the bacterium. One such example is from USA where the proportion of penicillin non-susceptible pneumococci declinded from 25% to 7% after data from 2006-2007 were analysed by using new penicillin breakpoints for non-meningeal invasive pneumococci that were published by CLSI in 2008 (10).

Macrolide and ketolide resistance

Dixon was the first to alert the scientific community to the development of erythromycin resistance in 1967, although the first anecdotal report on erythromycin resistance in pneumococcus occurred as early as in 1964, twelve years after the drug was launched for commercial use (211). In the 1980s the emergence of erythromycin resistance in pneumococci was evident: 0.3-6.3% of investigated pneumococci in the USA and 1.7-7.9% in Spain were reported to be resistant to erythromycin, while in France and in Belgium the respective proportion was already over 10% (211). Today, pneumococcal resistance to macrolides is a worldwide problem, although the prevalence of resistance varies greatly between countries, from 3% to 90% (89, 120, 158, 176, 187, 220, 229, 282, 293, 307, 317, 320, 336, 365). The highest prevalence of erythromycin resistance is in the Far East (~80%), followed by South Africa (~54%), southern Europe (~37%), northern Europe (~18%) and Latin America (~15%) (120). In the USA, erythromycin resistance in 2000-2004 was estimated at ~30% (187). Europe's hot spots of macrolide resistance are southern Europe and the Mediterranean region (prevalence ≥ 44%) (293). Countries with a low prevalence of macrolide

resistance (<11%) include Austria, the Czech Republic, Estonia, Norway, Portugal, Sweden, Russia, and the Netherlands (5, 107, 319). In Finland the prevalence was < 7% in 1999-2000 (273). Figure 2 presents the level of macrolide resistance among invasive pneumococci in European countries participating in the European Antimicrobial Resistance Surveillance System (EARSS) in 2005 (http://www.rivm.nl/earss).

So far, the telithromycin susceptibility of pneumococci has remained very good. At the time when telithromycin was undergoing clinical trials a worldwide longitudinal surveillance project to monitor the telithromycin susceptibility of respiratory pathogens was also introduced. This study was named PROTEKT (Prospective Resistant Organism Tracking and Epidemiology for Ketolide Telithromycin). Under the framework of this project, thousands of pneumococcal isolates have been collected since 1999 at regular intervals from dozens of countries all over the world. Susceptibility testing is performed in one laboratory with a standard method, which makes the estimation of trends reliable. So far it seems that the proportion of telithromycin non-susceptible pneumococci worldvide has remained at ≤ 0.3% and no increasing trend has been detected (120). In other publications the prevalence of telithromycin non-susceptibility has ranged from 0.02% to 3.6%, depending on the investigated pneumococcal population, breakpoints and methods used (24, 28, 40, 81, 109, 113, 169, 170, 182, 214, 215, 245, 342, 354). However, despite the satisfactory telithromycin resistance situation, several anecdotal reports have been published to date on clinical pneumococcal isolates showing a high level of resistance to telithromycin (106, 135, 165, 166, 289, 360, 362).

Penicillin resistance

Resistance to penicillin in a laboratory mutant pneumococcus was reported as early as the 1940's (211), but the first clinical pneumococci with elevated MICs to penicillin (MIC 0.1-0.2 mg/L) can be found in the report by Kislak and co-workers in Boston, USA, in 1965 (209). Two years later, a penicillin non-susceptible pneumococcus was isolated from a 25-year-old patient in Australia with a previous history of multiple antimicrobial treatments and hypogammaglobulinemia (151). This was followed by the emergence of penicillin-resistant isolates in New Guinea (211). Thereafter, penicillin resistance in pneumococci rapidly increased in many parts of the world. In 1977 there was an outbreak in South Africa caused by penicillin-resistant pneumococci (MIC 4-8 mg/L) that also were resistant to tetracycline, macrolides and chloramphenicol (56). In 1974-1984, already more than 10% of clinical pneumococcal isolates were penicillin non-susceptible in New

Guinea, Israel, Poland, South Africa, Spain, and in many states of the US (11), but pneumococci isolated from healthy carriers had even higher penicillin non-susceptibility rates of up to 36% (211). To date, the global penicillin non-susceptibility level has reached 36-37%, while the proportion of fully resistant strains is 23% (120). The highest penicillin non-susceptibility rates are in South Africa and the Far East (> 60-70%), followed by southern Europe (35-40%), Latin and North America (30-35%), and Australia (20-25%). The lowest penicillin non-susceptibility rates have been reported in northern Europe (10-15%) (120). Figures 3 and 4 present the penicillin non-susceptibility and penicillin resistance percentages of invasive pneumococci in European countries participating in EARSS in 2005 (http://www.rivm.nl/earss).

Fluoroquinolones and other antimicrobials

Fluoroquinolone resistance is still quite rare (≤ 2%) among pneumococci, although it has been observed to have increased within the past ten years (2, 58, 87, 91, 287). For instance, in Canada there were no fluoroquinolone-resistant pneumococci in 1993, but in 1997 their proportion was 1.7%. At the same time, fluoroquinolone prescriptions increased from 0.8 to 5.5/100 persons/year (58). In North America the proportion of ciprofloxacin-resistant pneumococci increased from 1.7 to 2.0% and levofloxacin-resistant pneumococci from 0.2% to 0.9% during 1997 to 1999 (194), while in 2001-2002 levofloxacin resistance had reached 1.1-1.3% in the USA (199). According to one international study that included seven countries worldwide, 1.3% (range 0-3%) of pneumococci were resistant to levofloxacin (246). A similar result was presented following another study (120). The highest fluoroquinolone resistance percentages have been reported in Italy (3%) (246), Hong Kong (3.8%) (167) and South Korea (3.8%) (312). Gatifloxacin resistance percentages are usually close to that of levofloxacin, but moxifloxacin resistance is usually lower due to its higher potency against pneumococcus (304). However, it must be noted that a pneumococcus showing diminished susceptibility to ciprofloxacin often has a mutation in a fluoroquinolone resistance target gene and is therefore also prone to developing resistance to newer quinolones.

Tetracycline resistance began to emerge as early as in the 1960s. By 1963-1964 in Australia, 25% of pneumococci isolated in hospitals were tetracycline resistant. Three years later in England, 18% of hospital isolates were reported to be tetracycline resistant, while the respective proportion in outpatients was 12% (211). Tetracycline resistance in the USA was around 15% in 2000-2004 (187) and in Europe ~ 20% in 2004-2005 (293). In Russia,

more than half of pneumococci isolated from carriers under five years old were observed to be resistant to tetracycline in 2001-2001, even though macrolide resistance was under 7% (323). This result may indicate differences in antimicrobial usage and therefore in selection pressure between the countries, but also differences in study designs or the origins of pneumococcal isolates.

Resistance to trimethoprim sulphonamide compounds was first detected in 1972 (211). It is now clear that the benefits of these drugs are being eroded by emerging resistance in pneumococci (173); the resistance to this antimicrobial class was 26.7% in Europe in 2004-2005 (293) and 24% in the USA (187). In Russia, the respective proportion was as high as 65% among children under 5 years old in day-care centres and orphanages (323).

Resistance to linezolide is extremely rare in pneumococci (347, 361). Resistance to vancomycin has not yet been observed in this bacterial species, although vancomycin-tolerant pneumococci have been described (163, 231, 255, 295, 324). The affinity of the drug to its binding site does not change and the MIC does not increase in such strains (163), but these isolates are able to escape lysis and killing by vancomycin, although the underlying mechanism is not yet clear (163, 324). Increased mortality has been noticed in meningitis patients if the causative organism is vancomycin-tolerant pneumococcus (295).

1.5 Clinical relevance of antimicrobial resistance

The clinical significance of antimicrobial resistance in pneumococci *in vitro* has been under continuous debate (119). Numerous reports have been published on this issue (30, 76, 96, 130, 163, 177, 205, 210, 224, 225, 271, 272, 299). Some studies have failed to find an association between antimicrobial resistance and the investigated outcome (i.e mortality or treatment failure) (321), especially when other risk factors and confounders have been taken into account (105, 168, 239, 262), while several others have documented such an association (222, 223, 253, 305, 341). Furthermore, there are dozens of case reports or series on pneumococcal infections in which treatment has failed due to antimicrobial resistance (41, 43, 54, 78, 82, 97, 98, 100, 101, 141, 174, 189, 203, 204, 206, 231, 260, 298, 318). The majority of these are macrolide or fluoroquinolone related, while reports of betalactam treatment failures are less frequent. On the basis of this evidence, it is clear that antimicrobial resistance has clinical significance. It is affecting

several parameters of morbidity by increasing the risk of breakthrough infections, the duration of illness and the costs of treatment. However, its effect on mortality is controversial (31, 239, 272, 364) or has been difficult to show due to factors such as the low statistical power in studies with small numbers of enrolled patients, difficulties in controlling for confounding factors (e.g co-morbidities, immune status, underlying diseases) or bias (e.g. in the selection of study subjects), retrospective study designs, and ethical reasons (299).

It has been suggested that low-level resistance, particularly in the case of betalactams and macrolides, has no clinical relevance (30, 31). Current evidence actually supports this claim for low-level betalactam resistance: infections caused by pneumococcal isolates showing intermediate susceptibility are treatable with betalactams as long as a higher dose and a more frequent dosing interval are used (18) in order to lengthen the time the drug concentration is above the MIC. However, proper pharmacodynamic parameters are difficult to achieve with tapering of the dose if a bacterial strain is fully resistant to penicillin (122). Studies show higher rates of mortality or suppurative complications in such infections (118, 239, 341) and they should therefore be treated with other agents (18) or combination therapy (247).

Regarding low-level macrolide resistance, such pneumococci usually have a relatively low macrolide MIC (2-16 mg/L), a concentration that should easily be achievable by macrolides at the infection site, and are therefore considered by some investigators to be treatable with macrolides (30). However, there is now increasing evidence that low-level macrolide resistance does have a clinical impact (174, 222, 305). For example, Lonks and co-workers described an increased risk of breakthrough bacteremia during macrolide therapy in patients with macrolide-resistant pneumococcus (222). They also documented breakthrough bacteremia in patients whose infection was caused by pneumococci showing a low-level resistant M phenoype (222). Another publication documented a case series of 122 patients with macrolide treatment failure (174). The majority of failures, including deaths, were in infections caused by pneumococci showing low-level macrolide resistance (174). A recent study demonstrated that treatment failures with macrolides are preceded by a low area under the inhibitory concentration-time curve (AUIC = AUC_{24}/MIC $\leq$ 10 for azithromycin, $\leq$ 31 for clarithromycin, and $\leq$ 53 erythromycin) and that patient factors such as co-morbidities were not in a key role in predicting the outcome (305). An earlier study revealed that pneumococci with azithormycin MICs > 2 mg/L are not eradicated by clinically achievable free drug concentrations in the blood or lungs,

regardless of the resistance genotype (305, 367). In addition, MICs of *mef*-carrying pneumococci have been documented invading to the right over time (107), increasing the clinical significance of this genotype.

1.6 Molecular typing methods to examine the epidemiology of drug resistant pneumococci

The epidemiological investigation of pneumococci has become necessary along with the rapid emergence of antimicrobial resistance. Conventional typing methods based on phenotypical characters, such as serotyping or antimicrobial profile (antibiogram) determination, are not sufficient to investigate the relatedness of different isolates. Therefore, several molecular typing methods, such as pulsed field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) have been developed and successfully used (103, 164, 234).

In PFGE the bacterial genome is cut with a restriction enzyme, usually into 10-30 fragments ranging from 10 to 800 kb. These fragments are then separated on an agarose gel using an electric current that pulses between three sets of electrodes, allowing DNA pieces to migrate through the gel PFGE has a high discriminatory power and is reproducible, but it is laborious and time consuming. In addition, interlaboratory comparison of the results can be challenging. Nevertheless, PFGE is widely used for investigating the genetic relatedness of several bacterial species. It is has been shown to be an effective tool for genotyping, especially if isolates have been collected within a relatively short period of time from a restricted geographical region, such as from suspected outbreaks (338).

Multilocus sequence typing (MLST), which was introduced in 1998, is currently considered as the gold standard for molecular typing of pneumococcal strains. The method is based on the sequencing of seven housekeeping genes or fragments of them, which allows the identification of pneumococcal clones and clonal complexes and also provides information on the genetic relatedness of isolates that differ at less than four of the seven loci (103) Apart from clonality studies, MLST can also be used to define pneumococcus species (149). The results of MLST are much easier to compare between the laboratories than PFGE results. On the other hand, in outbreak settings, PFGE has better discriminatory power compared to MLST.

The Pneumococcal Molecular Epidemiology Network (PMEN) was established in 1997 with the aim of characterizing, standardizing, naming and

classifying antimicrobial agent-resistant pneumococcal clones (234). In order to include a pneumococcal clone in the PMEN it has to fulfil the following criteria: 1) the clone must have a wide geographical distribution in a country or internationally; 2) the clone should have been present within a country for several years; 3) the clone should be resistant to one or more widely used antimicrobials; 4) data on the clone need to be published or in press prior to ratification of the network; and 5) a representative isolate should be provided in order to confirm that it differs from previously accepted PMEN clones. In addition the source must grant that the strain will be stored in the ATCC (American Type Culture Collection). By 2001, the PMEN described the nomenclature and characters of 16 drug-resistant pneumococcal clones concerning the worldwide spread of antimicrobial resistance. Examples of widely spread global clones include Spain23F – 1 and Taiwan19F-14 clones. The former was first described in Spain in the late 1980s and the latter in Taiwan in the 1990s. To date, these clones have spread to many European countries, the USA, South Africa, South America and the Far East (McGee et al., 2001). By August 2008, 26 PMEN clones had been described. Their nomenclature, MLST data and genetic relatedness are provided on the Multi Locus Sequence Typing Internet site (http://spneumoniae.mlst.net/pmen).

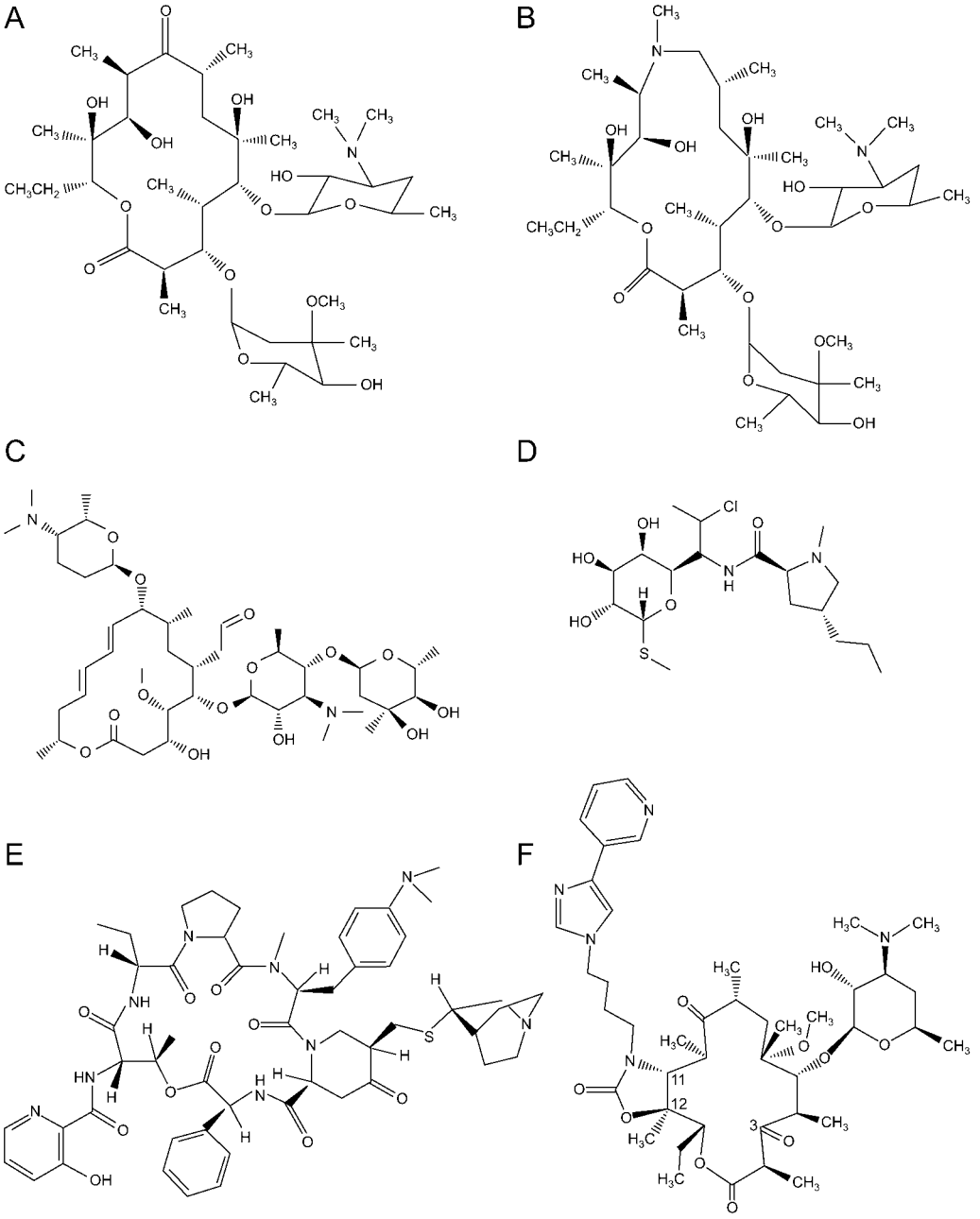

Figure 1. Chemical structure of A) erythromycin,14-membered macrolide: B) azithromycin, azalide, 15-membered ; C) spiramycin, 16-membered macrolide; D) clindamycin, lincosamide; E) quinupristin, streptogramin B; and F) telithromycin, ketolide.

Table 1. Overview of ribosomal mutations in clinical (C) and laboratory-derived (L) pneumococci and their MICs to erythromycin (ERY), clindamycin (CLI), spiramycin (SPI), quinupristin (QUI) and telithromycin (TEL) reported in macrolide-resistant pneumococci.

Mutation	Target**	ERY	CLI	SPI	QUI	TEL	Isolate type**	References
A2059C	Domain V	512	0.5-1	512	16-32	0.063-0.125	C	(275)
A2059G	Domain V	2-512	0.12-2	>100-512	1.56->128	0.01-0.063	C, L (ROX, AZM)	(52, 92, 108, 275, 332, 333)
A2058G	Domain V	1->100	0.12-16	6.25	16->128	0.01-0.5	C, L(AZM)	(52, 92, 108, 333)
A2058U	Domain V	>32	1			0.25-1	L(CLA)	(52)
C2611T	Domain V	≤ 0.12	0.5-1				C	(92)
C2611G	Domain V	0.5->512	0.06-1	0.78-16	64-128	0.015-0.5	C L(AZM)	(108, 275, 333)
C2610U	Domain V	0.06	0.5			0.008	L (CLA)	(52)
C2611U	Domain V	0.06-0.125	0.5-2			0.008-0.08	L(AZM, CLI)	(52)
C2611A	Domain V	0.78 - >64	0.20-0.25	1.56	50	0.01-0.06	C, L (AZM)	(108, 333)
A2059G+ G2057A	Domain V	>64	8	***		0.25	C	(108)
A2059G+ A2059C	Domain V	64	1	***		0.25	C	(108)

A2059C+ L4 G71R	Domain V, L4	>64	32	***		0.25	C	(108)
A2058U+ L4 I78V	Domain V 23, L4	>64	0.25-0.5	***		0.5	C	(108)
A2059G+ L22 G95D	Domain V, L22	64->64	1-2	***		0.6	C	(108)
A752 Deletion	Domain II	>32	1			4	L (CLA)	(52)
$_{69}$GTG$_{71}$to $_{69}$TPS$_{71}$	L4	>128->512	<-0.12-0.25	64	16	0.25	C	(92, 275, 332)
G$_{69}$C mutation	L4	0.05	0.20		3.12	0.006	L (AZM)	(333)
$_{67}$SQ$_{68}$ insertion	L4	0.20	0.05		3.12	0.01	L (AZM)	(333)
$_{71}$REKGTG$_{72}$ insertion	L4	6.25	0.05		25	3.12	C	(332)
$_{65}$WR$_{66}$ deletion****	L4	1	0.06		8	0.008	C	(361)
$_{68}$KG$_{69}$ deletion****	L4	2	0.06		8	0.008	C	(361)
K$_{68}$S mutation+ $_{69}$GTGR$_{72}$ deletion	L4	64	1	***		0.12	C	(108)
$_{68}$KE$_{69}$ to $_{68}$KEG$_{69}$insertion	L4	64	0.25	***		0.015	C	(108)

$_{64}$PWRQ$_{67}$ to $_{64}$P__Q$_{67}$ deletion	L4	2	0.25	*****	0.03	C	(108)
$_{69}$GT$_{70}$ to $_{69}$VP$_{70}$ change	L4	>64	0.25	***	0.03-0.06	C	(108)
$_{69}$GTG$_{71}$ to $_{69}$TPS$_{71}$ + V$_{88}$I	L4	>64	0.5-1	***	0.12	C	(108)
$_{108}$RTAHIT$_{110}$ tandem duplication	L22	1-32	0.03-0.5		1	C	(92, 108)
G$_{95}$D mutation	L22	0.25-1	0.03-0.125		0.12-0.125	L (ERY,ROX)	(52)
P$_{99}$Q mutation	L22	0.25	0.125		0.06	L (ERY)	(52)
A$_{93}$E+P$_{91}$S+ G$_{83}$E mutations	L22	0.5	0.03		0.25	L(TEL)	(52)
$_{92}$VRPR$_{93}$ insertion	L22	2	0.125	16	2	C	(275)
G$_{95}$D,G$_{71}$R mutations	L22, L4	0.25	0.06		0.06	L (AZM)	(52)
A$_{93}$E+ C2611A mutations	L22, Domain V	0.5	0.06		0.125	L (ROX)	(52)

* Domain V or II refers to domain numbers in 23S rRNA, L4 or L22 refers to ribosomal proteins. **abbreviations in paracenthesis after the laboratory isolates indicate which antimicrobial selected the type of mutation: clarithromycin (CLA), roxithromycin (ROX), otherwise as stated in the table legend.* **rokitamycin MICs 2->32 mg/L, ****non-susceptible to linezolide and chloramphenicol, *****rokitamycin MIC 1 mg/L

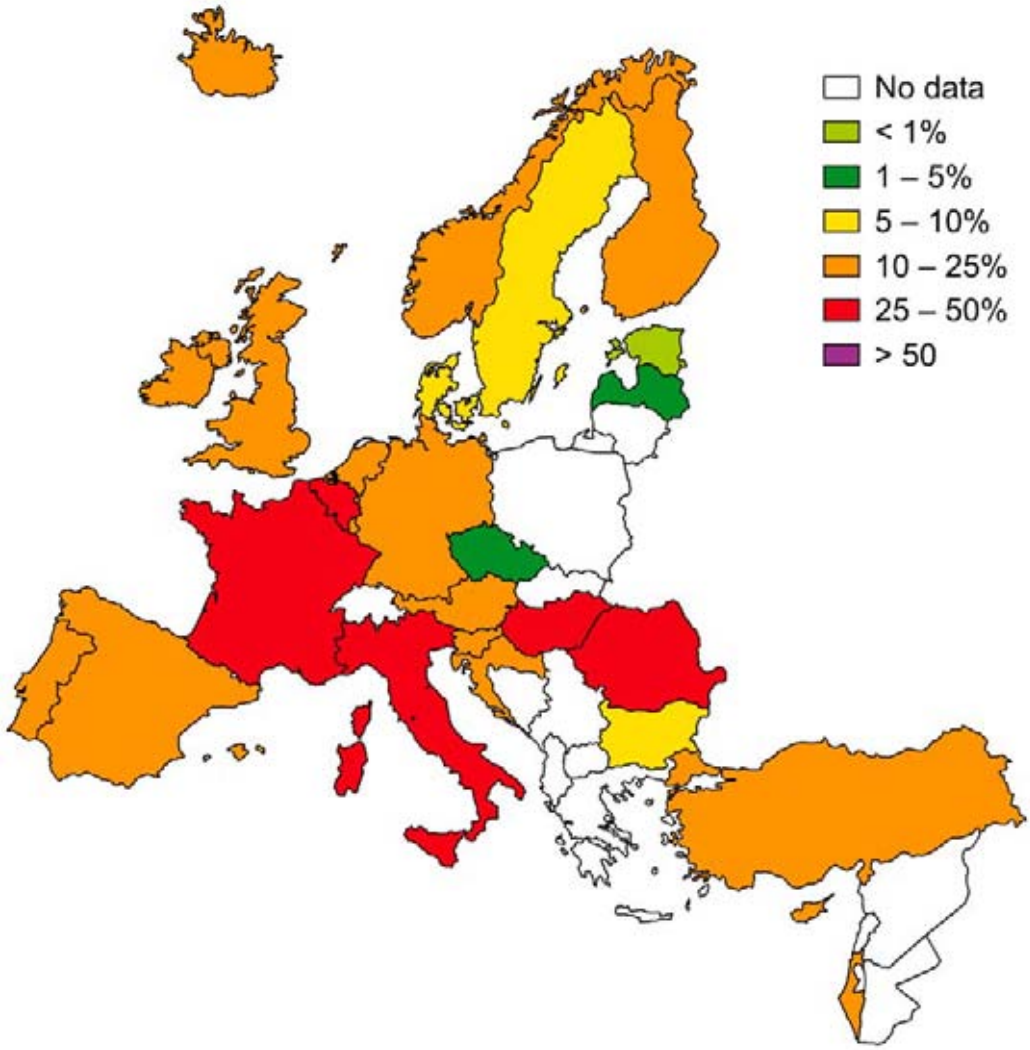

Figure 2. Proportion of erythromycin non-susceptible *S. pneumoniae* isolates in countries participating in EARSS in 2005.
(source: http://www.rivm.nl/earss/)

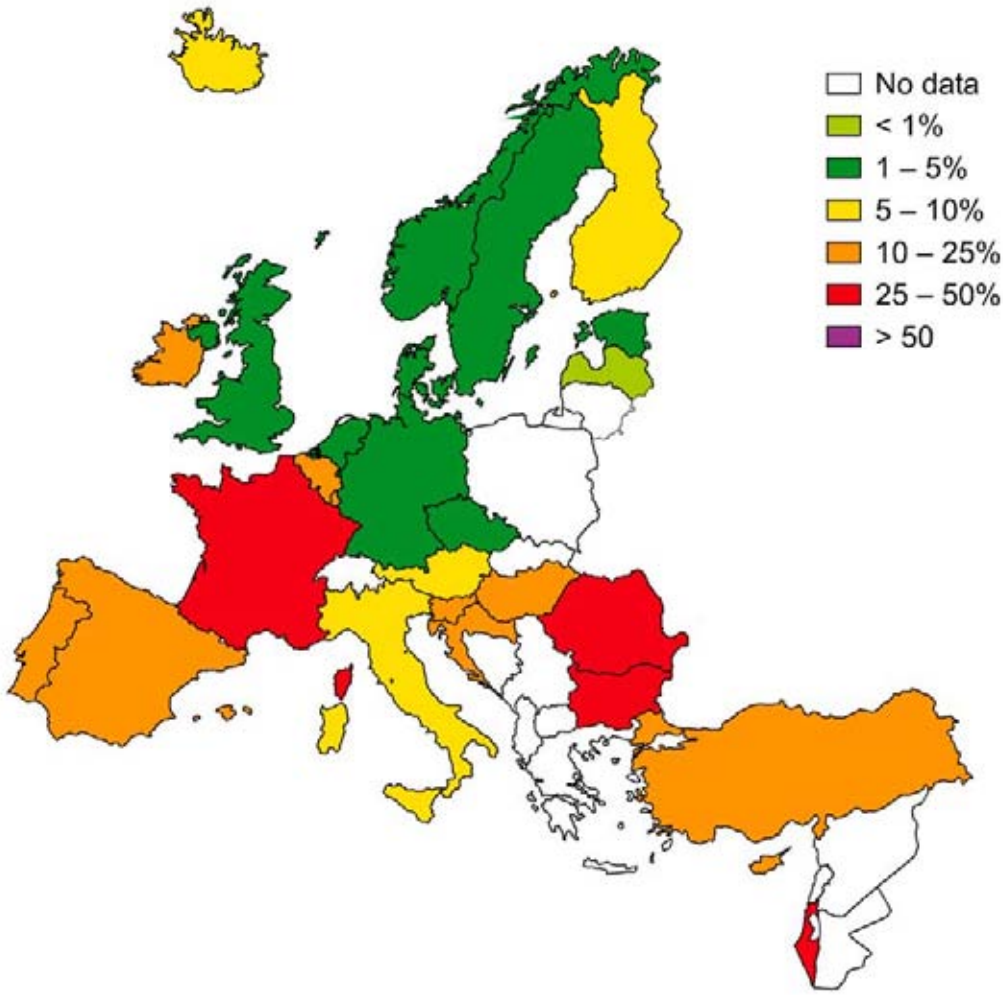

Figure 3. Proportion of penicillin non-susceptible pneumococci in European countries participating in EARSS in 2005.
(source: http://www.rivm.nl/earss/).

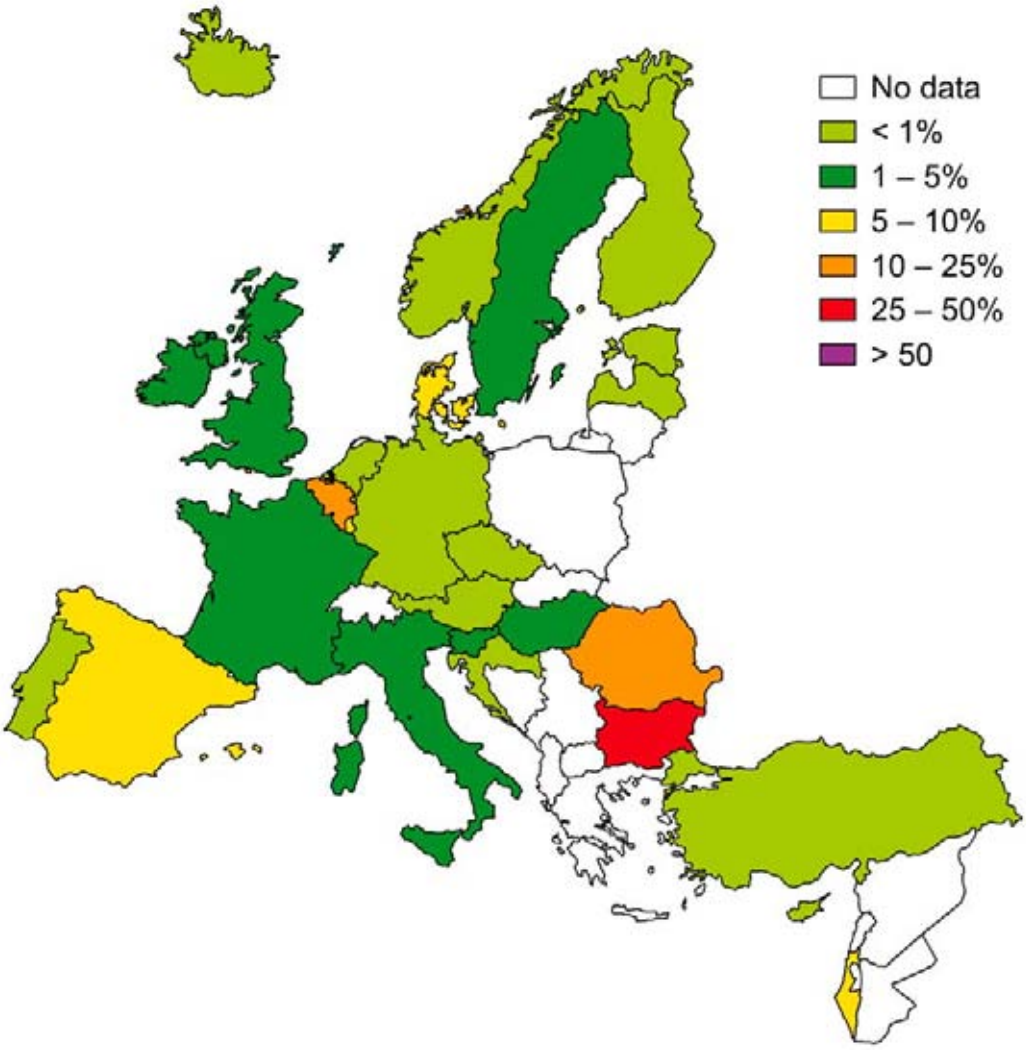

Figure 4. Proportion of penicillin-resistant pneumococci in European countries participating in EARSS in 2005.
(source: http://www.rivm.nl/earss/).

2. Aims of the study

The aims of this study were as follows:

1. To assess the prevalence of pneumococci non-susceptible to antimicrobials commonly used in Finland for treatment of pneumococcal infections, with special reference to macrolides;

2. To evaluate the level of telithromycin non-susceptibility among pneumococci prior to widespread telithromycin usage in Finland;

3. To determine the genetic basis of macrolide resistance in pneumococci

4. To examine the time trends of antimicrobial resistance in invasive pneumococci in Finland.

3. Materials and methods

3.1 Bacterial isolates

MILL-TELI02 collection (publications I-III): Clinical laboratories (n = 24) were asked to collect 50 consecutive clinical *S. pneumoniae* isolates and send the isolates to the Antimicrobial Research Laboratory of the National Public Health Institute (KTL) with the following information: laboratory ID, age and gender of the patient, date of isolation, and sample type. The participating laboratories belonged to the FiRe network (Finnish Study Group for Antimicrobial Resistance), which covers the whole country and all hospital districts. Nineteen laboratories out of 24 (79%) sent a total of 1007 *S. pneumoniae* isolates (2-68 isolates per laboratory) between May and December 2002. Of the 1007 isolates, 878 (87%) were from non-invasive and 129 (13%) from invasive infections. Non-invasive isolates were derived from ears (n = 367), sinuses (n = 205), sputum or trachea (n = 107), eyes (n = 84), pus (n = 67) and bronchus (n = 48). The first three publications of this thesi are based on all these isolates (I) or a subset of the isolates of this collection (II-III).

Invasive pneumococcal collection 2002-2006 (publication IV): The bacterial population in this study consists of all invasive *S. pneumoniae* isolates (n = 3571) collected by clinical laboratories in Finland during the period of 2002-2006. Of these, 3423 (96%) were blood isolates and 148 (4%) were cerebrospinal fuild (CSF) isolates. Clinical laboratories sent the isolates to the Culture Collection of the National Infectious Disease Register of the KTL. The following information was provided with each isolate: date of isolation, laboratory ID, specimen type (blood or CSF) and the date of birth of the patient. The number of isolates per year ranged from 574 to 753. The number of bacterial isolates sent to the Culture Collection corresponded 97.8% to the number of blood and CSF pneumococcal isolates notified to the National Infectious Disease Register during the same period.

3.2 Identification of pneumococci and serotyping

The primary identification of *S. pneumoniae* was performed using conventional methods in clinical laboratories and was based on colony morphology, typical greenish alpha-haemolysis on 5% sheep blood agar (Oxoid Ltd., Basingstoke, Hampshire, United Kingdom), and optochin susceptibility (Optochin disk, Oxoid, Ltd.). Identification was further confirmed with a Slidex Pneumo-Kit (bio-Mérieux SA, Marcy l'Etoile, France), serotyping, or molecular methods when necessary. Serotyping was performed by latex agglutination for the neutral serogroups/types 7 and 14, followed by counterimmunoelectrophoresis with pneumococcal omni, pool, group/type and factor sera (Statens Serum Institut, Copenhagen, Denmark). The Quellung reaction was used as a confirmation method when needed (161, 207).

3.3 Testing antimicrobial susceptibility

Agar plate dilution method: The minimum inhibitory concentration (MIC) of an antimicrobial was determined by an agar plate dilution technique according to a previously described method (275). Briefly, a bacterial suspension corresponding to a 0.5 McFarland standard was prepared from a fresh overnight culture. It was further diluted 1:10 to reach a final inoculum concentration of 10^7 CFU/ml. An aliquot of each inoculum was applied to the surface of Mueller-Hinton II agar (Becton Dickinson Microbiology Systems, Cockeysville, Md., USA) with a 3 mm multipoint inoculator (Denley, Mast Labs, Liverpool, England) to reach a final inoculum 10^4 CFU per spot. Agar plates were prepared as instructed by the CLSI guidelines. Testing media were supplemented with 5% (v/v) of sheep blood for all antimicrobials except for testing SXT susceptibility, for which 2% (v/v) defibrinated horse blood was used. Plates were incubated at 35 °C supplemented with a 5% CO_2 atmosphere for 18-20 hours. The MIC was recorded as the lowest antimicrobial concentration that completely inhibited bacterial growth. Telithromycin powder was kindly provided by Sanofi-Aventis (Romainville, France). Other antimicrobials were purchased from their respective manufacturers: penicillin, tetracycline, erythromycin, clindamycin, ceftriaxone, SXT (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), azithromycin (Pfizer Inc., New York, N.Y.), levofloxacin (Hoechst Marion Roussel [Sanofi-Aventis], Romainville, France), and meropenem (AstraZeneca, London, UK). Breakpoints given for pneumococci by the CLSI (66) were used, with the exception of azithromycin, for which the

following breakpoints were used: S ≤ 1 mg/L, I = 2 mg/L, R ≥ 4 mg/L (Table 3). *S. pneumoniae* ATCC 49619 and *S. aureus* ATCC 29213 were used as quality controls in each run.

Further testing of telithromycin susceptibility (publications II, IV): In addition to agar plate dilution testing, telithromycin susceptibility testing was performed for 210 erythromycin non-susceptible pneumococci with a known macrolide resistance determinant [88 with *erm*(B), 4 with *erm*(B)+*mef*(E), 104 with *mef*(A/E), and 14 with a mutation] and 47 randomly-selected erythromycin-susceptible pneumococci by the CLSI disk diffusion method using 15 μg telithromycin disks (Oxoid Ltd., Bansingtone, Hampshire, England). Breakpoints for telithromycin susceptibility in disk diffusion test were as follows: R ≤ 15 mm, I 16-18 mm, S ≥ 19 mm. (66). If colonies were detected inside the growth inhibition zone (indicating heterogeneous resistance to telithromycin), one colony was picked (termed the zone isolate), streaked onto a blood agar plate and incubated for 18-20 h at 35 °C in a 5% CO_2 atmosphere. After confirming that the zone isolate was the same species, it was stored at -70 ˚C until susceptibility testing was performed with the same method. Susceptibility testing was repeated 3-5 times for isolates showing heterogeneous resistance to telithromycin in the disk diffusion test. Telithromycin MICs of the isolates showing heterogeneous resistance to telithromycin and their respective zone isolates were also determined by the CLSI broth microdilution in a normal atmosphere (66) and by agar plate dilution in 5% CO_2 to compare the MICs of the isolates with these two methods. The ATCC strains mentioned above were used as quality controls.

Investigating the stability of the telithromycin resistance (publication II): A serial passage method was used to test the stability of telithromycin resistance of three zone isolates (zone isolates, see paper II p. 1855). Isolates were cultured on 5% sheep blood agar plates (35 ˚C in 5% CO_2). After overnight incubation, sub-isolates were cultured again onto a new blood agar plate. The procedure was repeated five times on consecutive days. The susceptibility of telithromycin of each five sub-culture isolates was tested by the disk diffusion method as described above.

3.4 Detecting macrolide resistance determinants

Polymerase chain reaction PCR (publications I, IV): Detection of macrolide resistance genes, *mef*, *erm*(B), and *erm*(A) – [*erm*(TR) subclass] - was performed by a multiplex PCR method as described previously (123). Separate PCR reactions were run to differentiate efflux gene subclasses *mef*(A) and *mef*(E) in *mef*-positive strains and to detect the presence of *msr*(D). Briefly, bacterial cells for PCR were harvested from fresh overnight culture and lysed by incubating for 10 minutes at 95 ºC to isolate the total DNA. AmpliTaq DNA polymerase enzyme (Applied Biosciences, Foster City, Ca.) was used for all PCR reactions. The $MgCl_2$ concentration was 2 mM in multiplex PCR and 1.5 mM in other PCR reactions. Multiplex PCR included initial denaturation at 94 °C for 10 min, followed by 39 cycles of denaturation at 94 °C for 30 s, annealing at 51 °C for 30 s and elongation at 72 °C for 1 min. In other PCR reactions the annealing temperature was 56 °C for detecting *mef*(A) and *msr*(D), and 58 °C for *mef*(E). The number of cycles was 35; otherwise conditions were similar to those in multiplex PCR. All PCR reactions were run with a Whatman Biometra thermocycler (Biometra, Goettingen, Germany). Strains known to be positive for the respective determinant were included with every run as positive controls and a PCR mixture with nuclease-free water instead of bacterial DNA was used as a negative control. The primers are listed in Table 2.

Bacterial isolates investigated using this method included 261 pneumococci of the MILL-TELI02 (I). Of these, 217 were non-susceptible to erythromycin, three were erythromycin susceptible (MIC $\geq$ 0.5 mg/L) but had low-level resistance to clindamycin (MIC 2-4 mg/L) and azithromycin (MIC 4-8 mg/L), and 41 isolates, which were randomly selected, were susceptible to all macrolides, clindamycin and telithromycin. Of the invasive pneumococcal isolates from the 2002-2006 collection (IV) we screened 223 erythromycin non-susceptible pneumococci for the presence of macrolide resistance genes.

Detection of mutations conferring macrolide resistance (publications I, II): Mutations at positions 2058-2059 and 2611 of domain V and in loop 35 of domain II of 23S rRNA were detected by a pyrosequencing technique (296, 297). Mutations in genes encoding 50S ribosomal proteins L4 and L22 were searched for by sequencing as previously described (183, 333). Primers used for pyrosequencing and sequencing are listed in Table 2. Sequencing was

performed using an ABI Prism BigDye Terminator Kit (Applied Biosystems) according to the manufacturer's instructions. The sequences were analysed using Vector NTI Suite 8 software (InforMax Inc, Bethesda, MD, USA). Comparison of sequences was performed with the sequence similarity and homology searching tools (Proteomes & Genomes Fasta) using a WWW interface to the European Bioinformatics Institute (EBI) genome database.

Ribosomal mutations Domain V and II of 23S RNA and in genes encoding ribosomal proteins L4 and L22 were screened from those macrolide-resistant isolates that did not carry resistance genes (n = 18, MILL-TELI02 collection, publication I). In addition, one isolate with *mef*(E) and one with a double mechanism of *mef*(E) and *erm*(B) were investigated. These mutations were also screened for some selected telithromycin resistant isolates as well as their zone isolates (publication II).

Sequencing of the 23S rRNA gene and *erm*(B): The whole 23S rRNA gene as well as the *erm*(B) gene with its promoter region (beginning 280 base pairs upstream from the start of the ErmB methylase protein coding region) were amplified and sequenced from some selected telithromycin resistant isolates in order to investigate possible mutations in these genes (see publication II). Primers and conditions for 23S rRNA PCR and sequencing have been described elsewhere (183, 333); *erm*(B) sequencing primers are listed in Table 2. For *erm*(B) amplification the PCR mixture had a total volume of 50 µl, containing 10 pmol of each *erm*(B) PCR primer, 2 U of Ampli Taq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA), 1.5 mM of $MgCl_2$, 5 µl of -10 x PCR buffer, 10 pmol of nucleotides, and 5 µl of DNA template. The PCR conditions were as follows: initial denaturation at 94 ˚C for 15 min, followed by 35 cycles of denaturation at 94 ˚C for 30 s, annealing at 56 ˚C for 30 s, and extension at 72 ˚C for 60 s. Sequencing and data analysis were performed as mentioned above.

3.5 Investigating the genetic relatedness of the isolates

Pulsed-field gel electrophoresis analysis (publication III): Pulsed-field gel electrophoresis (PFGE) analysis was performed for all telithromycin resistant isolates, three zone isolates (see publication II, page 1855) and 22 randomly selected *erm*(B)-positive telithromycin-susceptible isolates from the MILL-TELI02 collection. Published methods were followed in the preparation of

bacterial genomic DNA for PFGE analysis (228) and in performing PFGE (219). Restriction endonuclease *Sma*I (New England BioLabs, Inc., USA) was used for digestion. DNA fragments were separated in a 1.2% PFGE-agarose gel with a CHEF DR^{R} III system (Bio-Rad, Hercules, CA, USA). Gels were stained with 1 µg/ml ethidium bromide solution and photographed under UV light. Dendrograms and cluster analysis were performed using the BioNumerics software package (Applied Maths, Inc., Austin, TX). Strains having PFGE profiles with ≥ 85% similarity (UPGMA, Dice coefficient) were considered as clones.

Multilocus sequence typing (publications III, IV): MLST was performed for all isolates with heterogeneous resistance to telithromycin and all invasive isolates from 2002-2006 that were penicillin resistant according to a slight modification of a published method (103). Briefly, bacterial isolates were cultured on to 5% sheep blood agar and grown overnight at +35 °C in 5% CO_2. Bacterial genomic DNA was isolated from 1 µl of fresh overnight culture with a Dneasy Tissue Kit (QIAGEN GmbH, Germany). Approximately 450 base pair fragments of seven housekeeping genes were amplified by PCR: *aro*E (shikimate dehydrogenase), *ddl* (D-alanine-D-alanine ligase), *gdh* (glucose-6-phosphate dehydrogenase), *gki* (glucose kinase), *rec*P (transketolase), *spi* (signal peptidase I) and *xpt* (xantine phosphoribosyltransferase). The primers used are listed in Table 2. The PCR products were purified using the QiaQuick PCR purification kit (QIAGEN GmbH, Germany). The amplicons were sequenced on both strands. The sequences were analyzed using the program Vector NTI version 10 (Invitrogen Corporation, California, US) and the results were compared with the MLST database, after which the sequence types (STs) were assigned. The STs were compared with those in the MLST database and the clonal complex group for each strain was determined by E-burst analysis. The method and database are available via the MLST website (www.mlst.net).

Table 2. Primers used in the study

Target	Use**	Sequence(5'-3')		Reference
		Forward	Reverse	
mef(A/E)	multiplex-PCR	GGCAAGCAGTATCATTAATCAC	GACTGCAAAGACTGACTATAG	(123); this work
erm(B)	multiplex-PCR	GAAAARGTACTCAACCAAATA	AGTAAYGGTACTTAAATTGTTTAC	(326)
erm(A)	multiplex-PCR	CTTGTGGAAATGAGTCAACGG	TTGTTCATTGGATAATTTATC	(275)
mef(E)	PCR	GGGAGATGAAAAGAAGGAGT	GCTATAAAATGGCACCGAAAG	(77); this work
mef(A)	PCR	TGGTTCGGTGCTTACTATTGT	CCCCTATCAACATTCCAGA	(64)
msr(D)	PCR	CAGTTGGACGAAGTAACTCTG	CTCTTACGTTCTTCCTCTTTC	(77); this work
L4 ribosomal protein	PCR/seq	AAATCAGCAGTTAAAGCTGG	GAGCTTTCAGTGATGACAGG	(333)
L22 ribosomal protein	PCR/seq	GCAGACGACAAGAAAACACG	ATTGGATGTACTTTTTGACC	(333)
Domain V 23S rRNA, position 2058-2059	PCR	TAAGGTAGCGAAATTCCTTGTCG	CGACCGCCCCAGTCAAACT**	(143)
	Pyroseq	GGTTACCCGCGACAGGACGG		(143)
Domain V 23S rRNA, position 2611	PCR	TGGGTTCAGAACGTCGTGAGA	GCGGTAAGTCCACTCTGGTC**	This work
	Pyroseq	CGTGAGACAGTTCGGTC		
Whole *erm*(B)	PCR	GAAGCAAACTTAAGAGTGTG	GCTAGGGACCTCTTTAGCTT	This work

Table 2. Primers used in the study

	Seq	CAGTGATTACGCAGATAAATA	GACACGAATGTTCAGTTTTA	This work
	Seq	CCTAAACCAAAAGTAAACAG	TCTCGATTGACCCATTTTGA	This work
Loop 35 of domain II	PCR	GCGCCTTAGTATCATGACGTAGA	AATGTCGACGCTAGCCCTAAAG**	This work
	Pyroseq	CGCTACCCACAAGTCA		This work
	Pyroseq	CGTGAGACAGTTCGGTC		This work
Shikimate dehydrogenase (*aro*E)	MLST	CGTTTAGCTGCAGTTGTTGC	CCCACACTGGTGGCATTAAC	(102)
D-alanine-D-alanine ligase (*ddl*)	MLST	TTGCCATGGATAAAATCACGA	CGCGCTTGTCAAAACTTTCC	Bichon B personal comm; (29)
Glucose-6-phosphate dehydrogenase (*gdh*)	MLST	ATGGACAAACCAGC(G/A/T/C)AG(C/T)TT	GCTTGAGGTCCCAT(G/A)CT(G/A/T/C)CC	(103)
Glucose kinase (*gki*)	MLST	GGCATTGGAATGGGATCACC	TCTCCCGCAGCTGACAC	(103)
Transketolase (*rec*P)	MLST	GAATGTGTGATTCAATAATCACC	TTCGATAGCAGCATGGATGG	(102)
Signal peptidase I (*spi*)	MLST	CGCTTAGAAAGGTAAGTTATG	AGGCTGAGATTGGTGATTCTC	Bichon B, personal comm.
Xantine phosphor-ribosyltransferase (*xpt*)	MLST	TTATTAGAAGAGCGCATCCT	AGATCTGCCTCCTTAAATAC	(103)

* Seq – sequencing, Pyroseq – pyrosequencing; **Primer biotinylated

3.6 Data analysis

Descriptive analysis of antimicrobial resistance included the calculation of resistance percentages as well as the MIC_{50}, MIC_{90}, and MIC range when appropriate. Resistance percentages were calculated by using the intermediate breakpoint as a cut-off if not otherwise stated, except for penicillin, for which the proportions of penicillin non-susceptibility (PNSP, PEN I and R isolates) and penicillin resistance (PEN R) were calculated. An isolate showing non-susceptibility to ≥ 3 different antimicrobial classes was defined as multiresistant. Resistance percentages were also calculated according to the age group, hospital district and University Hospital District of the cases. The University Hospital Districts were Helsinki-Uusimaa, (Southern Finland), Turku (South-Western Finland), Tampere (Central and Mid-Western Finland), Kuopio (Eastern Finland) and Oulu (Northern Finland). The age groups were 0-2 years, 3-15 years, 16-64 years and ≥ 65 years. The two first mentioned age groups were combined for statistical testing due to the small number of cases among 3-15 years in the publication IV. The chi-squared test was used to compare resistance percentages in non-invasive and invasive pneumococci (publication I) and the proportion of ceftriaxone-resistant isolates in 2005-2006 (publication IV). The Mann-Whitney test was used to compare erythromycin MICs between *mef*(A) and *mef*(E) isolates (publication I), and the Poisson regression was used for testing the statistical significance of the trends of antimicrobial resistance over the time (publication IV). The logarithm of the number of isolates for each day was incorporated as an offset. In the model, time was considered as a continous variable and district and age group were categorical variables. Risk ratio estimates (representing the relative change in the risk for a strain being resistant to an antimicrobial within a one-year period) and 95% CIs were calculated, while a P value ≤ 0.05 was considered statistically significant.

4. Results

4.1 Occurrence and trends of antimicrobial resistance

MILL-TELI02 collection (publication I)

Of the 1007 *S. pneumoniae* isolates collected in 2002, 21.5% were non-susceptiblel to erythromycin, 11% to clindamycin and 2.6% to telithromycin. The proportion of isolates non-susceptibile to penicillin, tetracycline and sulphatrimethoprim was 12.1%, 14.4% and 26.8%, respectively. The ceftriaxone non-susceptibility rate was 2.1% if the meningitis breakpoint was used, but only 0.1% with the non-meningitis breakpoint. Less than 1.5% of isolates were resistant to fluoroquinolones and only two isolates non-susceptible to linezolide. No vancomycin-resistant isolates were detected. MIC data (MIC_{50}, MIC_{90}, range) and the distribution of the 1007 pneumococcal isolates among separate susceptibility categories are presented in Table 3. Multiresistance was detected in 10.5% of isolates (n = 106), with 6.7% (n = 67) of isolates resistant to four or more and 2.3% (n = 23) to five more antimicrobial classes. The most common multiresistant phenotype was ERY-PNSP-TET-SXT (n = 34). Erythromycin resistance was equally frequently detected among non-invasive and invasive isolates. However, isolates from non-invasive infections were more frequently non-susceptible to penicillin and multiresistant (Table 4). Bronchus and ear isolates were the most resistant and isolates from the eyes the most susceptible (Table 5). Resistance to macrolides ranged among hospital districts from 6 to 38%, penicillin non-susceptibility from 2 to 23%, and tetracycline resistance from 4 to 33%. Figure 5 illusrates macrolide and penicillin non-susceptibiliy percentages by hospital district. Resistance percentages are presented according to age group and university hospital district in Tables 6 and 7, respectively.

Table 3. MIC data (MIC50, MIC90, min-max) and distribution of isolates (%) among susceptibility categories (S - susceptible, I - intermediate, R - resistant) for 1007 pneumococcal isolates in Finland, 2002

Antimicrobial agent	mg/L			% of strains			Breakpoints*		
	MIC50	MIC90	min-max	S	I	R	S ≤	I	R ≥
Erythromycin	0.125	64	0.016 - ≥128	78.5	0.4	21.1	0.25	0.5	1
Clindamycin	0.125	2	0.008 - ≥128	89.0	0.6	10.4	0.25	0.5	1
Azithromycin	0.5	≥128	0.063 - ≥128	77.7	0.4	21.9	1	2	4
Spiramycin	1	32	0.125 - ≥128				ND**		
Telithromycin**	0.031	0.5	0.008 – 8	97.4	1.9	0.7	1	2	4
Penicillin	0.016	0.125	0.008 – 4	87.9	9.2	2.9	0.063	0.125	2
Ampicillin	0.031	0.125	0.008 – 8				ND		
Cephalexin	0.125	0.5	0.016 – 16				ND		
Cefuroxime	0.031	0.25	0.008 – 16	93.4	1.4	5.2	0.5	1	2
Ceftriaxone	0.016	2	0.008 – 2	97.9	2.0	0.1	0.5	1	2
Meropenem	0.008	0.125	0.008 – 1	95.7	3.9	0.4	0.25	0.5	1
Levofloxacin	1	2	0.126 – 16	98.8	0.9	0.3	2	4	8
Moxifloxacin	0.25	0.5	0.063 – 8	98.6	1.1	0.3	1	2	4
Tetracycline	0.25	32	0.063 – 64	85.6	0.2	14.2	2	4	8
Trimethoprim-sulfamethoxazole	0.25	4	0.125 – 64	73.2	13.6	13.2	0.5/9.5	1/19-2/38	4/76
Linezolide	1	2	0.125 – 4	99.8		0.2	2		
Vancomycin	0.25	0.25	0.125 – 1	100.0			1		

*All breakpoints are acccording to CLSI standards, except for azithromycin, for which breakpoints were determined according to the MIC distribution. Ceftriaxone breakpoints in the table are meningitis breakpoints. Ceftriaxone breakpoints for non-meningitis isolates are as follows: S ≤ 1 mg/L, I 2 mg/L, R 4 ≥ 4 mg/L. With non-meningitis breakpoints, 99.1% of isolates were susceptible and 0.1% intermediate. **ND - breakpoints not determined by CLSI. **Telithromycin non-susceptibility rates presented here should be interpreted with caution because these breakpoints are for the CLSI microdilution method for a normal atmosphere.

Table 4. Comparison of erythromycin non-susceptibility, penicillin non-susceptibility and multiresistance in non-invasive and invasive pneumococcal isolates in Finland, 2002.

Resistance profile	Non-invasive (n = 878)	Invasive (n = 129)	P	χ^2
	%			
ERY (I+R)	22.8	20.9	0.6	0.22
PEN (I+R)	13.6	5.4	0.014	6.02*
Multiresistance (ERY-PEN-TET)**	8.2	1.6	0.012	6.36*

*statistical testing performed with Yates correction, **ERY – erythromycin, PEN - penicillin, TET –tetracycline. I – intermediate, R – resistance

Table 5. Percentage of resistance* to erythromycin (ERY), clindamycin (CLI), levofoxacin (LEV), tetracycline (TET) and trimethoprim-sulfamethoxazole (SXT) of pneumococcal isolates according to specimen origin in Finland, 2002.

Specimen origin	ERY	CLI	PEN	LEV	TET	SXT
	%					
Ear (n = 367)	27	14	16	1	17	31
Sinuses (n = 205)	20	10	11	2	14	28
Blood or CSF (n = 129)	20	9	5	0	10	19
Sputum/trachea (n = 107)	18	8	12	1	18	20
Eyes (n = 84)	11	7	5	5	5	20
Pus (n = 67)	16	6	19	0	9	50
Bronchus (n = 48)	29	13	15	0	29	37

*Including both I (intermediate) and R (resistant) isolates

Table 6. Resistance* of pneumococcal isolates to erythromycin (ERY), penicillin (PNSP) and tetracycline (TET) according to age group in Finland, 2002

Age group (years)	No. of isolates	ERY	PNSP	TET
		%		
0 - 2	384	28.1	17.7	19.3
3 - 15	224	14.7	8.5	7.1
16 - 64	305	17	8.2	10.8
≥ 65	94	24.5	10.6	23.4

* Includes both intermediate and resistant isolates

Table 7. Percentage resistance of pneumococcal isolates according to University Hospital District in Finland, 2002

University Hospital District	No. of isolates	Resistance %		
		ERY	PNSP	TET
Helsinki-Uusimaa	395	19.5	13.4	13.7
Kuopio	109	25.7	11.9	14.7
Oulu	167	28.7	19.8	20.4
Tampere	238	13.9	4.6	8.8
Turku	98	31.6	12.2	20.4
Total	1007	21.5	12.1	14.4

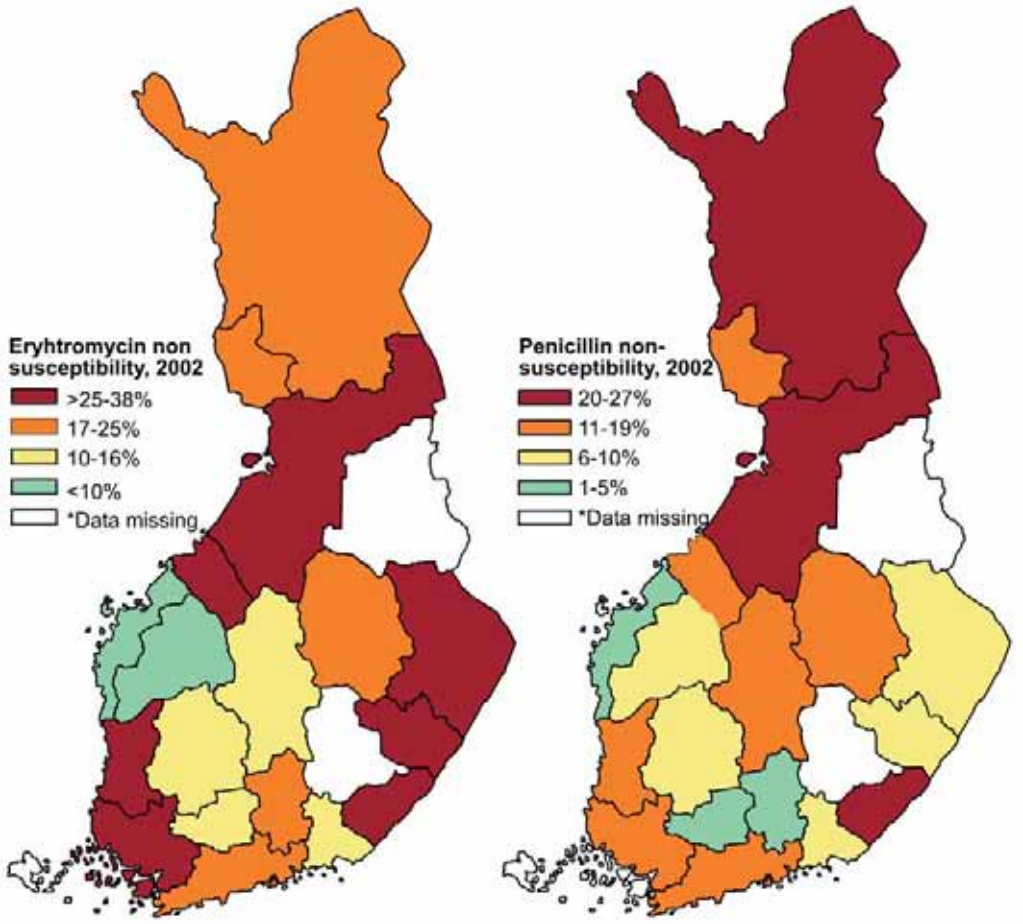

Figure 5. Erythromycin and penicillin non-susceptibility of pneumococcal isolates in hospital districts in Finland, 2002 (MILL-TELI02 collection)

Antimicrobial resistance in invasive pneumococci 2002-2006 (publication IV)

Erythromycin resistance ranged from 16% (2002) to 28% (2006) and increased significantly during the study period ($p < 0.0001$). The proportion of penicillin non-susceptible isolates doubled from 8% to 16% ($P = 0.0002$) and proportion of penicillin-resistant isolates increased from 0.8% to 3.7% ($p = 0.03$). Tetracycline resistance remained stable. The proportion of multiresistant isolates increased from 3.7% (2002) to 5.1% (2006), but this change was not significant ($p = 0.72$) (Table 8). The resistance figures for CSF isolates were similar to those for blood isolates. Ceftriaxone non-susceptibility was 2.9% in 2005 and 3.7% in 2006 (chi-squared test, $p = 0.36$), but MICs were low: only two isolates (0.3%) had an MIC of 2 mg/L in 2006, but none in 2005. Five isolates in 2004 (1.7%), three in 2005 (0.5%) and one in 2006 (0.1%) were resistant to levofloxacin. All levofloxacin-resistant pneumococci were isolated from older patients (median 69 years). Figure 6 illustrates the development of antimicrobial resistance during the study period. The figure also includes previously published antimicrobial resistance data from the period of 1999-2000 (273) and unpublished data from 2001.

The highest erythromycin resistance rates were detected among pneumococci isolated from 0- to 2-year-old children: in 2006, 45.8% of isolates were resistant to erythromycin in this age group. Time trend risk ratio estimates for erythromycin non-susceptibility by age group were as follows: 0-15 years RR 1.12, ($p = 0.01$); 16-64 years RR 1.11 ($p = 0.003$), and ≥ 65 years RR 1.16 ($p < 0.001$). For penicillin non-susceptibility, the respective estimates are: 0-15 years RR 1.11 ($p = 0.14$), 16-64 years RR 1.22 ($p < 0.001$) and ≥ 65 years RR 1.15 ($p = 0.01$). The proportion of penicillin resistant isolates increased significantly in 16-64 years (RR 1.53, $p < 0.001$), but the trend was not statistically significant in any other age group. Resistance development over time according to age group can be seen in the publication IV, Figure 1. At the university hospital district level, a significant increase in erythromycin resistance was observed in the Tampere district, from 7% to 29% (RR 1.35, 95% CI 1.20-1.52, $p < 0.0001$) and Oulu, from 20% to 28% (RR 1.12, 95% CI 1.00-1.25, $P = 0.04$). The increase of penicillin non-susceptibility was statistically significant in Tampere district, from 5% to 15% ,(RR 1.25, 1.07-1.45, $p = 0.004$) and Kuopio district, from 5% to 20% ,(RR 1.30, 1.04-1.61, $p = 0.02$). PEN resistance increased significantly in Tampere, from 1% to 5% ($P = 0.002$), and in Kuopio, from 0% to 10% ($P = 0.006$).

Table 8. Antimicrobial resistance in invasive pneumococci in Finland at the beginning (2002) and the end (2006) of the study period and the relative change in resistance per year according to the Poisson regression model.

Antimicrobial*	Resistance percentages in the first and last years of the study period		Relative change per year, ratio	95% Confidence intervals		p-value
	2002	2006		Lower	upper	
ERY	16.3	27.9	1.12	1.06	1.18	<0.0001
PNSP	8.0	16.4	1.15	1.07	1.23	<0.0001
PEN R	0.8	3.7	1.18	1.02	1.36	0.03
TET	9.8	11.5	1.00	0.93	1.08	0.99
ERY-PNSP-TET	3.7	5.1	1.02	0.91	1.14	0.72

*ERY- erythromycin I+R isolates; PNSP - penicillin non-susceptibile I+R isolates; PEN R - penicillin resistant isolates; TET - tetracycline I+R isolates; multiresistance (ERY-PNSP-TET)

Resistance by serotype (publication IV)

The most common serotypes in decreasing order of frequency in 2006 were 14 (20%), 4 (11%), 6B (9%), 23F (7%), 3 (6%), 7F (6%), 19F (5%) and 9V (5%). The most prominent change was detected in serotype 14, the proportion of which increased from 14% to 20% during 2002-2006. Serotype 14 isolates were most frequently non-susceptible to erythromycin or penicillin. Table 9 presents the overall frequency of different serotypes and the proportion of erythromycin and penicillin non-susceptible isolates in different serotypes during the study period.

Table 9. The frequency of different serotypes and the rate of antimicrobial resistance according to serotype among invasive pneumococci (n=3571) in Finland, 2002-2006

Serotype frequency			Non-susceptibility (%)	
Serotype	N	%	ERY*	PNSP**
14	551	15.4	57.8	36.3
4	405	11.3	4.7	3.2
23 F	272	7.6	8.5	6.6
9 V	268	7.5	41.8	13.4
3	243	6.8	2.9	3.3
6 B	250	7.0	40.2	20.8
7 F	228	6.4	2.6	1.3
19 A	163	4.6	41.7	11.0
18 C	156	4.4	3.2	1.9
19 F	155	4.3	26.5	20.6
9 N	125	3.5	12.8	4.0
22 F	119	3.3	5.9	2.5
6 A	108	3.0	10.2	3.7
12 F	97	2.7	2.1	ND
11 A	51	1.4	5.9	ND
Others	380	10.6	8.4	1.6

*ERY – erythromycin non-susceptibility, include both intermediate and resistant isolates. **PNSP – penicillin non-susceptible pneumococci, include intermediate and resistant isolates.

Clonality results for penicillin-resistant invasive pneumococci 2002-2006 (publication IV)

Multilocus sequence typing was performed for 88 penicillin-resistant invasive pneumococci in 2002-2006. Table 10 summarises the results of MLST typing of these pneumococci. See Table 3 in publication IV, for more detailed results. In summary, a total of 25 sequence types were found that distributed into ten clonal lineages (clonal complexes, CC). In addition, two singletons (ST3248, ST3249) were observed that have not previously been described outside Finland. The most common clonal complex was CC156, accounting for 61% of all penicillin-resistant isolates, followed by CC271 (10% of the isolates) and CC81 (9% of the isolates). This means that the majority of the penicillin-resistant pneumococci in this study were representatives or single to triple locus variants of the following PMEN clones: Spain^{9V} ST156, Taiwan^{19F} ST236, Spain^{23F} ST81, and England^{14} ST9. Other international clones or closely related strains occurring among penicillin-resistant pneumococci were England^{14} ST9, Spain^{6B} ST90, Sweden^{15A} ST63, and Sweden^{4} ST205.

Table 10. Occurrence of different clonal complexes and sequence types among penicillin-resistant invasive pneumococci (n = 88) in Finland, 2002-2006

Clonal complex	N	%	Secuence types (n)	PMEN clone and its variants*
CC156	54	61.4	ST156 (29), ST2306 (11) , ST143 (7), ST2916 (4), ST2918 (1), ST3247 (1), ST671 (1)	Spain^{9V} ST156 + its SLVs, DLVs and TLVs
CC271	9	10.2	ST2917 (3), ST271 (2), ST236(2), ST2694 (1), ST3245 (1)	Taiwan^{19F} ST236 + its SLVs and DLVs
CC81	8	9.1	ST961 (5), ST81 (2), ST3250 (1)	Spain^{23F} ST81 + its SLVs
CC15	4	4.5	ST13 (4)	SLV of England^{14} ST9
CC146	4	4.5	ST90 (3), ST3246 (1)	Spain^{6B} ST90 + its DLVs
CC63	2	2.3	ST2678 (2)	SLV of Sweden^{15A} ST63
CC205	1	1.1	ST205 (1)	Sweden^{4} ST205
CC496	2	2.3	ST496 (2)	None
CC138	1	1.1	ST138 (1)	None
CC460	1	1.1	ST461 (1)	None
Singleton	1	1.1	ST3248 (1)	None
Singleton	1	1.1	ST3249 (1)	None

PMEN = Pneumococcal Molecular Epidemiology Network, *SLV = single locus variant, DLV = double locus variant, TLV = triple locus variant

4.2 Macrolide resistance determinants (publications I, IV)

The most frequent macrolide resistance mechanism in 2002 (publication I) was the *mef* gene, which was present in 49% (107/220) of macrolide-resistant isolates, while the respective frequency of *erm*(B) was 41% (Table 11). *msr*(D) was present in all *mef*-positive isolates. Altogether, 89% of *mef* isolates (n = 95) had the *mef*(E) subclass and 11% (n = 12) had *mef*(A). Erythromycin MICs of the *mef*(A)-positive strains were higher than those of the *mef*(E) strains (geometric means 32 and 10.9 mg/L, respectively, p = 0.002, Mann-Whitney U-test). Mutation was detected in 16 isolates, of which 14 isolates had no other known resistance factor. Six new ribosomal protein mutations were found that had not previously been described in clinical isolates. Of these, four mutations were in the L4 protein ($_{68}E_{69}$, $_{68}GQK_{69}$, $T_{94}I$, $V_{205}G$) and two in the L22 protein ($R_{22}C$, $A_{101}P$). Pneumococcal strains with mutations and their phenotypes are presented in the Table 1 of the publication I on page 4181. Figure 7 (right panel) shows the distribution of erythromycin MICs according to the macrolide resistance determinant.

Table 11. Macrolide resistance mechanisms in pneumococci in Finland, 2002

Mechanism	n	%
mef(E)	95	43.2
mef(A)	12	5.5
erm(B)	90	40.9
mef(E) + *erm*(B)	5	2.3
erm(A)	1	0.5
Mutation only*	14	6.4
Unknown	3	1.4

*Altogether, 16 strains had a mutation, but a macrolide resistance gene was present in two of them

In the invasive pneumococcal collection from 2002-2006 the *mef* gene was also the most common macrolide resistance determinant, and was detected in 56% (n = 125) of the investigated isolates (n = 223). *erm*(B) was present in 31% (n = 69) of the isolates and both *mef*(E) and *erm*(B) were carried by two isolates (0.9%). Of the *mef*-positive isolates which were further investigated (n = 60), 72% (n = 43) were *mef*(E) and 28% (n = 17) *mef*(A). All *mef*(A) isolates belonged to serotype 14 and were susceptible to penicillin. In 28 isolates the macrolide resistance mechanism remained unknown. In contrast to the MILL-TELI02 collection there were no significant differences in ERY MICs of the invasive pneumococci between the *mef*(A) and *mef*(E) isolates, although the geometric mean of the ERY MIC was slightly higher in *mef*(A)-positive isolates compared to that of *mef*(E) isolates (36.2 mg/L vs. 28.1 mg/L). There was no indication of a right shift in ERY MICs in *mef*-positive strains.

4.3 Telithromycin resistance

MIC, zone size distribution and resistance genes (publication I)

In the MILL-TELI02 collection, telithromycin MICs ranged from 0.008 to 8 mg/L; 2.6% of isolates had an MIC ≥ 2 mg/L (Table 3). The isolates formed two distinct populations (Figure 7, left panel), of which larger population (83% of isolates) had an MIC range from ≤ 0.008 to 0.063 mg/L and the smaller population (17% of isolates) from 0.125 to 8 mg/L. Telithromycin MICs in relation to the macrolide resistance determinant are illustrated in Figure 7 and respective zone sizes are presented in publication II, Table 1, page 1856.

Heterogeneous resistance to telithromycin (publications II, IV)

In disk diffusion testing, 26 pneumococcal isolates produced one to several clearly visible colonies inside the growth inhibition zone, indicating heterogeneous resistance to telithromycin (publication II). The typical growth pattern of such isolates can be seen in publication II, Figure 1, p. 1856. All isolates showing colonies inside the inhibition zone were classified as resistant to telithromycin, regardless of the zone size. Susceptibility testing demonstrated that the phenomenon was repeatable, but the number of colonies inside the inhibition zone varied. All isolates with heterogeneous resistance to telithromycin were *erm*(B) positive and two of them also carried

mef(E). We also subcultured colonies growing inside the growth inhibition zone and tested their telithromycin susceptibility. Figure 1 of publication II on p. 1856 illustrates the typical narrow inhibition zone of a zone isolate. In these isolates, telithromycin resistance was observed to be constant and did not diminish in five serial passages. The mean diameter of the inhibition zone of the pneumococci (publication II) with heterogeneous resistance was 20.9 mm (range 15 – 24 mm), whilst the respective diameter in sub-isolates grown from colonies picked from inside of the inhibition zone (i.e. zone isolates, n = 26) was 8.3 mm (range 6 – 19 mm). Of the zone isolates, 23 were classified as resistant and two as intermediate. One zone isolate had an inhibition zone diameter of 19 mm.

Among invasive pneumococci collected in 2002-2006, heterogeneous telithromycin resistance was detected in 9 out of 128 (7%) erythromycin resistant isolates tested with the disk diffusion test.

Comparison of telithromycin MICs between agar plate and broth microdilution methods (publication II)

Telithromycin MICs of isolates showing heterogeneous telithromycin resistance in the disk diffusion test and their respective zone isolates were determined concurrently by the CLSI broth microdilution method in ambient air and by the agar dilution in a 5% CO_2 atmosphere. The telithromycin MIC_{50} and MIC_{90} of the isolates showing heterogeneous resistance to telithromycin were 2 and 4 mg/L (range 0.063 to 8 mg/L), respectively, when measured by the agar dilution method (Table 12). With the broth microdilution method the respective values were 0.125 and 1 mg/L (range 0.063 – 2 mg/L). Thus, all but one isolate with heterogeneous resistance to telithromycin would have been classified as susceptible according to current breakpoints with the broth microdilution method in a normal atmosphere. The telithromycin MIC_{50} and MIC_{90} of the zone isolates was 32 mg/L and 64 mg/L, respectively, according to the agar dilution method in 5% CO_2, whilst they were 4 and 8 mg/L with CLSI broth microdilution in ambient air. Three zone isolates failed to grow with the latter method (Table 12).

Table 12. Comparison of telithromycin MICs of isolates showing heterogenous resistance and their respective zone isolates determined with the agar dilution and CLSI broth microdilution methods

Parameter	*erm*(B) isolates with heterogeneous resistance*		zone isolates**	
	Agar dilution method in 5% CO_2	CLSI Broth microdilution method	Agar dilution method in 5% CO_2	CLSI Broth microdilution method
	n = 26	n = 24***	n = 26	n = 23***
	Telithromycin MIC (mg / L)			
Geometric mean	1.89	0.15	33.8	2.87
Min – max	0.063-8	0.063-2	4-64	0.125-8
MIC_{50}	2	0.125	32	4
MIC_{90}	4	1	64	8
No. of isolates with MIC ≥ 2 mg/L (%)	21 (81%)	1 (4%)	26 (100%)	21 (91%)

* Isolates having a visible inhibition zone but individual colonies detected inside this zone

** Zone isolates were derived from individual colonies from inside the inhibition zone

***Some isolates did not grow with the microdilution method in a normal atmosphere

Tracing the possible mechanism for telithromycin resistance (publication II)

The *erm*(B) gene and its promoter region (beginning 280 base pairs upstream from the start of the ErmB methylase protein coding region) were sequenced for three telithromycin-resistant isolates and their respective zone isolates (# 547, 415, 1022 and 547V, 415V 1022V). Genes encoding 50S ribosomal proteins L4 and L22 of the above-mentioned six isolates were also sequenced and ribosomal mutations in domains V and II of 23S rRNA were detected by pyrosequencing. In addition, the whole 23S rRNA gene of one telithromycin-resistant isolate and the respective zone isolate (#547, 547V) were sequenced. The nucleotide sequences of *erm*(B) and its promoter area, positions 2058-2059 and 2611 of domain V, and loop 35 of domain II of 23S rRNA, as well as genes encoding 50S ribosomal proteins L4 and L22, were identical to heterogeneously telithromycin-resistant isolates and zone isolates. No mutations known to confer resistance in the promoter area of *erm(*B), in 23S rRNA genes or in genes coding for L4 or L22 were detected. The consensus sequences of 23S rRNA for 547 and 547V had 99.97% and 99.83% similarity with published genomic 23S rRNA sequences of *S. pneumoniae* strains TIGR4 and R6, respectively. Thus, no mechanisms known to be associated with telithromycin resistance were identified in this study.

Genetic relatedness of the telithromycin-resistant isolates (publication III)

The clinical characteristics, PFGE profiles and sequence types (STs) of the heterogeneously telithromycin resistant pneumococci (n = 26) are summarized in Paper III, Table 1, p. 1885. The isolates originated from both invasive and non-invasive infections in twelve hospital districts of Finland. These areas cover all parts of Finland except for Lapland. The patients' mean and median ages and age ranges were 16.5, 2 and 0-84 years, respectively. Fifteen isolates were obtained from female patients and ten from males. Penicillin non-susceptibility (MIC $\geq$ 0.125 mg/L) was less frequent among *erm*(B)-positive isolates showing heterogeneous resistance to telithromycin (4/26) than among *erm*(B)-positive but telithromycin-susceptible isolates (50/66) ($p < 0.0001$, Fisher's exact test). Tetracycline resistance (MIC $\geq$ 4 mg/L) alone was as frequently observed among *erm*(B)-positive

telithromycin-resistant isolates as among *erm*(B)-positive telithromycin-susceptible pneumococci. None of the isolates was resistant to levofloxacin or linezolid. Serotype 19A was the most frequent serotype. The PFGE dendogram is presented in Paper III, Figure 1, page 1886. Overall, *erm*(B)-positive telithromycin-susceptible pneumococci were more heterogeneous compared to *erm*(B)-positive telithromycin-resistant isolates. Twenty-six telithromycin resistant isolates were distributed into seven distinct PFGE types, of which type A was the most frequent with 19 telithromycin-resistant isolates. Three subtypes were detected among those isolates with the type A pattern, but 14 isolates belonged to subtype A1. Four telithromycin susceptible isolates had a PFGE pattern closely related to telithromycin-resistant strains. Three zone isolates had an identical PFGE pattern to their respective parent isolates. Twenty-three telithromycin-susceptible *erm(*B) isolates were distributed among twelve distinct PFGE types. MLST results were in accordance with the PFGE results: telithromycin-resistant isolates had seven distinct sequence types. Of these, ST2248, 2306 and 2307 were novel types. The other four sequence types were ST193, 133, 271 and 271, of which ST193 was the most prevalent and corresponded to PFGE type A.

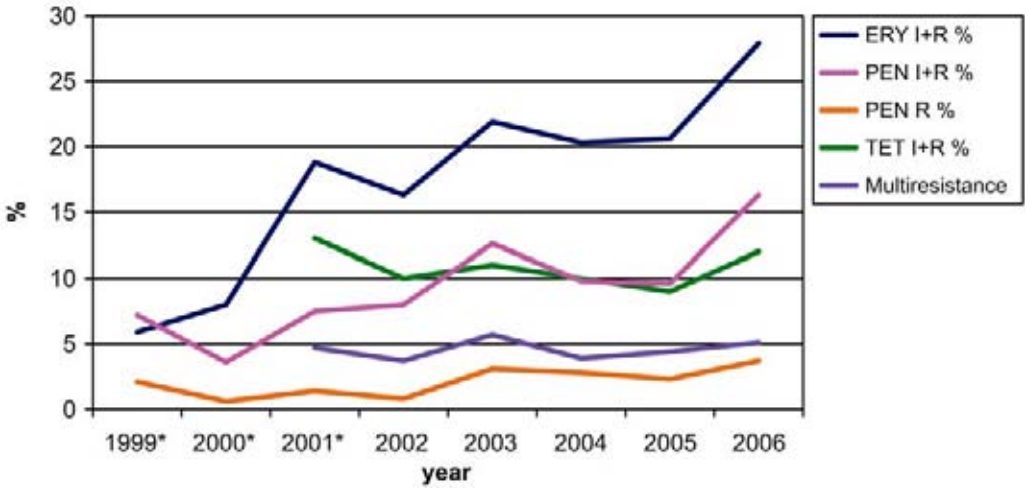

Figure 6. Resistance development in invasive pneumococci in Finland during 1999-2006. Percentage resistance is indicated on the y axis. The number of isolates tested per year ranged from 360 to 753. *The results from 1999-2000 are from a previously published report by Pihlajamaki et al. (AAC, 2002) and the data from 2001 are previously unpublished. ERY – erythromycin, PEN – penicillin, TET – tetracycline, I – intermediate, R – resistant; multiresistance: non-susceptibility to ERY-PEN-TET.

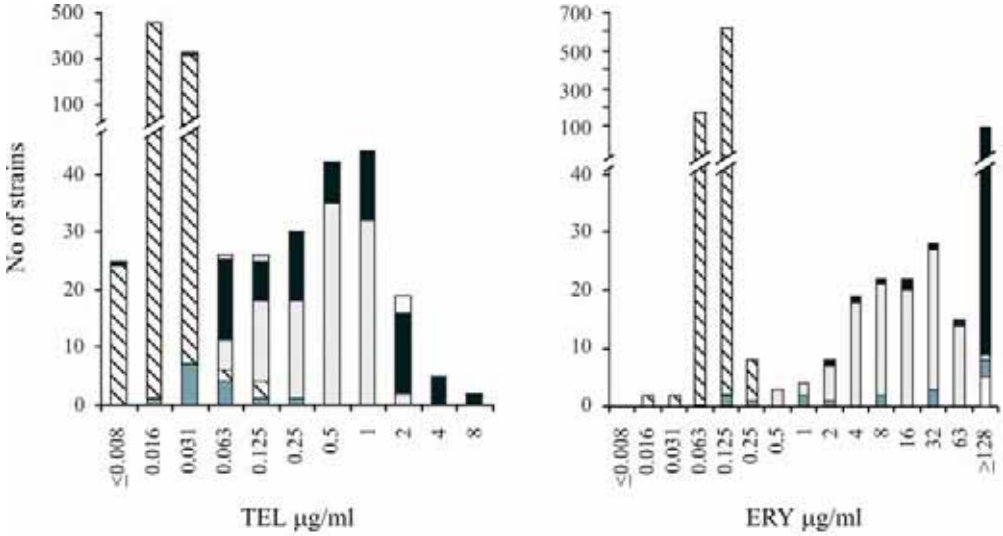

Figure 7. Left panel: distribution of telithromycin MICs, right panel: distribution of erythromycin MICs of 1007 clinical pneumococci. Bar symbols: oblique – strains susceptible to macrolides; dark grey – strains with mutations; pale gray – strains with *mef*(A) or *mef*(E); black – strains with *erm*(B) or *erm*(A); white – strains with double mechanism *erm*(B)+*mef*(E).

5. Discussion

5.1 Macrolides

Resistance

Of the 1007 *S. pneumoniae* isolates collected in 2002 from a variety of infections in Finland (MILL-TELI02 collection), one fifth were resistant to erythromycin. Azithromycin resistance percentage was slightly higher (22.3%) than the respective figure for erythromycin. The minor difference can be explained by the presence of some mutated strains that showed

azithromycin non-susceptibility but were erythromycin susceptible. Therefore it can be concluded that the azihtromycin breakpoints adjusted for our method worked well. Azithromycin and clarithromycin resistance percentages are usually close to that of erythromycin (181, 292) and could be considered as indicators of erythromycin resistance. The proportion of clindamycin-resistant isolates was half of that for erythromycin-resistant isolates in this study. This result reflects the distribution of macrolide resistance genes in Finland, since the major resistance mechanism was drug efflux encoded by the *mef* gene, which does not cause resistance to clindamycin (218). The highest antimicrobial non-susceptibility figures were observed among pneumococci isolated from from children aged 0-2 years, which is in accordance with many other studies (44, 90, 107). This is most likely due to the high antimicrobial consumption among this age group (13).

Pneumococcal isolates of non-invasive origin are often more resistant to antimicrobials than invasive isolates (137, 193, 266, 340). Apart from erythromycin resistance, similar observations were recorded in our study. The difference might be explained by the fact that bacteriological cultures are more often performed in the early phase of infection in the case of invasive infections. Varying resistance figures in pneumococci of different origin can also reflect differences in the use of antimicrobials for treating various infections. This is supported by the fact that pneumococcal isolates from the eye were most commonly susceptible to erythromycin but most frequently resistant to fluoroquinolones. Topical fluoroquinolones, on the other hand, are commonly used to treat eye infections, while macrolides are not.

Results based on invasive pneumococci isolated in 2002-2006 demonstrated a significant increase in erythromycin resistance from 16 to 28%. Compared to the period from 1999-2000, the increase in erythromycin resistance was almost five-fold (273). Erythromycin resistance rates similar to Finland (20-30%) have been reported from Germany (22%), the UK (24%), Switzerland (29%) (107) and Ireland (22%) (291). Finland has a higher prevalence of resistance among pneumococci than its neighbouring countries. For example, in Norway approximately 4% of over 9000 pneumococcal isolates collected in 1995-2005 were non-susceptible to erythromycin, although an increase in resistance from 1.7% in 1995 to 10.6% in 2005 was detected during the study period (319). Sweden, on the other hand, is an example of a country where the proportion of erythromycin-resistant pneumococci has remained low. This is most likely because of a successful conservative antimicrobial policy

leading to a reduced consumption of antimicrobials, especially macrolides (243). According to Swedish national surveillance data, less than 5% of pneumococci were resistant to erythromycin in 2007 (http://www.srga.org//ResNet). This also applies to Estonia and Russia (5, 107, 214). Countries with a low prevalence of macrolide resistance (<10%) also include the Czech Republic, Portugal, the Netherlands, and Denmark (107, 293). In Great Britain, slightly higher erythromycin resistance rates have been detected, ranging from 9 to 14% depending on the data source (221). Today, the highest erythromycin resistance prevalences in Europe (44-53%) occur in France, Greece, Italy and Hungary, followed by Spain (34%) (107). Globally, almost 40% of pneumococci were erythromycin resistant in 2003-2004 (107), the highest resistance rate being in the Far East (~80%) and the lowest in Northern Europe and Latin America (~15-18%) (120). In the USA, the prevalence of erythromycin resistance in 2000-2004 was ~30%, ranging from 17% (south-west region) to 39% (south-central region) (187). Regional differences were also observed in our studies. In 2002, erythromycin resistance varied among university hospital districts from 14 to 32%, being lowest in Tampere and highest in Turku according to the results from the MILL-TELI02 collection. The results for invasive pneumococci in were in accordance with resistance levels recorded in 2002, although erythromycin resistance was somewhat lower. Thereafter, resistance increased most rapidly in Tampere district, followed by Kuopio and Oulu districts. By 2006, regional differences had declined, although the highest prevalence of resistance was still recorded in Turku.

Methodological considerations

In our studies, MIC susceptibility testing was performed with the plate agar diffusion method in a 5% CO_2 atmosphere because 5-10% of pneumococcal isolates do not grow adequately in air (65, 117). CLSI guidelines, however, recommend broth microdilution and incubation in a normal atmosphere for pneumococcus. Both methods have been demonstrated to reliably to separate susceptible and resistant pneumococcal populations (117, 182, 273), although CO_2 supplementation is known to elevate the MICs of pneumococci to macrolides and ketolides by approximately a one to three dilution, the effect of CO_2 being perhaps most prominent for azithromycin (39, 65, 81, 192, 348). However, because nearly all our pneumococcal isolates exceeding the macrolide or clindamycin non-susceptibility breakpoints had a macrolide resistance mechanism, we regard our results as reliable.

It is known that CO_2 mainly affects pneumococci with a macrolide resistance mechanism, while only a slight increase in macrolide MICs is detected in wild type isolates (117, 182). For example, Fasola and co-workers observed that 5% of erythromycin-resistant and up to 43% of clindamycin-resistant pneumococci were misclassified into the wrong susceptibility category when incubation was performed in ambient air, but when incubated in CO_2 the strains were clearly separated into susceptible and resistant categories, and the susceptible strains remained within the susceptible range (117). This result was also achieved when the incubation time in ambient air was extended from 24 hrs to 48 hrs. The authors concluded that the result was mainly due to the enhanced growth of resistant strains and earlier expression of resistance in CO_2 rather than impaired antimicrobial activity, because only a small change in the pH of the culture media occurred in CO_2 (117). Macrolides, ketolides and clindamycin are alkaline substances, the activities of which diminish in low pH conditions (191). CO_2 has no significant effect on the MICs of other antimicrobials (61, 65, 180).

Macrolide resistance mechanisms

Efflux and methylase

The *mef* gene was observed to be the most frequent macrolide resistance gene in Finland. The macrolide resistance gene distribution in pneumococci was very similar to that observed in 1999-2000 by Pihlajamaki and co workers (273). Finland's macrolide resistance gene distribution is more comparable with North America and Scotland than continental Europe (8, 107, 170, 187, 188, 358). In the USA, 66% of macrolide-resistant pneumococci carried the *mef* gene in 2000-2004 (187), while in Canada the proportion of *mef*-positive isolates from 1998-2004 was 47% (358). The *mef* gene is also the prevailing macrolide resistance mechanism in Australia, Germany, Greece, Ireland, the UK and the USA (107, 120). A recent study revealed that the erythromycin resistance MICs of *mef*-positive isolates are invading to the right (120), but this was not observed in our study, perhaps due to the low number of *mef* isolates in successive years and therefore the low statistical power.

On the global level, *erm*(B) appears to be the most common macrolide resistance determinant in pneumococci. According to a recent study, its prevalence was 55% while the respective figure for the *mef* gene was 31% (107, 120). Countries with a high prevalence of the *erm*(B) gene include

Belgium (91%), France (90%), Spain (88%), Hungary (82%), Poland (80%), China (77%), Italy (56%) and Japan (58%) (107). Pneumococci carrying both *mef* and *erm*(B) have rapidly emerged within the last few years. The emergence of a dual resistance mechanism in pneumococci was first documented in 1999 in South Africa, where McGee and co-workers observed that 31% of macrolide-resistant pneumococci carried both of these genes and that the isolates were clonally related (233). The global prevalence of these isolates nearly doubled from 7% in 1999 (115) to 12% in 2004 (107). Today countries with a high prevalence of the double resistance mechanism in pneumococci (20-46%) include South Africa, South Korea, the USA, China and Australia (107). Our results suggest that the proportion of such isolates is rare in Finland. A dual mechanism was detected in five strains of macrolide-resistant pneumococci in the MILL-TELI02 collection, and in only two isolates (0.9%) of the invasive pneumococcal collection in 2002-2006. Nevertheless, the spread of this genotype is of great concern and warrants careful monitoring. The vast majority of strains with a dual mechanism are multiresistant and clonally related, belonging to the widespread Taiwan$^{19F\text{-}14}$ clone (107, 187, 233) Moreover, it is alarming that pneumococci of this type are frequently isolated in 0- to 2-year-old children (107), which can increase the risk of treatment failures among this age group. The efflux determinant occurring simultaneously with the *erm*(B) gene seems to exclusively be the *mef*(E) subtype (8, 115). This was also the case in Finnish isolates. Interestingly, in a very recent congress abstract the presence of *mef*(A) was described together with *erm*(B) in pneumococci in Germany (34).

erm(A) [subclass of *erm*(TR)] is a rare methylase mechanism in pneumococcus, but is frequently found in *Streptococcus pyogenes* (114, 201). In our study we found only one pneumococcal strain carrying this gene. Apart from Finland, *erm*(A) has been detected in individual pneumococcal strains from Greece, Belgium, the USA and Italy (51, 111, 330, 344).

mef subtypes

According to our results, the majority of *mef* genes (70-90%) were of the *mef*(E) subtype and 10-30% were *mef*(A). Among other geographical regions, *mef*(E) appears to dominate in Canada, the USA, South Africa and Eastern Europe, whilst both *mef*(A) and *mef(*E) occur in Mediterranean and Western European countries (77). In Scotland, Italy and Norway, *mef*(A) has been observed to be the most common *mef* subtype, and its spread was clearly related to the emergence of the ST9 clone in these countries (8, 69, 86, 302).

The ST9 PMEN clone is also known as the erythromycin-resistant but penicillin-susceptible England[14]-9 clone (234). *mef*(A) also seems to be frequent in Germany (34). We observed that invasive pneumococcal isolates carrying *mef(*A) possessed serotype 14 and shared an identical antibiogram. The latter applies also to *mef*(A) isolates of the MILL-TELI02 collection, but serotype information was not available. These results suggest that *mef*(A) strains are closely related and might be considered as indirect evidence of the presence of a penicillin-susceptible ST9 clone in Finland. Previous data have shown that the majority of reported ST9 clone members posses serotype 14 (69). However, the presence of serotypes 3, 19A, 23F, 31, and 33A among the isolates belonging to ST9 suggest that this ST is prone to capsular switch (69). Tetracycline resistance in *mef*(A)-positive pneumococci has seldom been reported (69), but was observed in one strain of our study. It seems that the tetracycline resistance gene *tet*(M) occurs together with *mef*(A) in pneumococci (69), unlike in *Streptococcus pyogenes*, in which *tet*(O) has been reported with *mef*(A) (134).

According to some investigators, the erythromycin MICs of *mef*(E) isolates are higher than those of *mef*(A) isolates (8), but this has not been confirmed (69). In our study a significant difference in ERY MICs between *mef*(A) and *mef*(E) isolates was observed in the first set of pneumococcal isolates, but not in the invasive isolates, although the geometric mean of the ERY MIC was to some extent higher in *mef*(A)-positive pneumococci of the latter collection.

Ribosomal mutations

The proportion of macrolide-resistant pneumococci carrying a ribosomal mutation was 6.4% (MILL-TELI02 collection). Of the erythromycin-resistant invasive isolates, 10% did not carry a macrolide resistance gene. Although these isolates were not screened for ribosomal mutations, phenotype data suggested the presence of mutation. These results are in accordance with Pihlajamaki and co-workers, who reported that 10% of invasive pneumococci had a ribosomal mutation or unknown mechanism in Finland in 1999-2000 (273). For some reason, macrolide resistance due to mutations seems to be more common in Finland than in many other countries. According to a European multicentre study including 8 European countries, the prevalence of ribosomal mutations was only 1% among erythromycin-resistant pneumococci (287). In Germany, 1.3% of 399 macrolide-resistant isolates had a ribosomal mutation (290), while in the Netherlands the respective figure was 5% (251) and in Belgium only 0.4% (344). However, the last-mentioned Belgian result

was in contrast to the study by Farrell et al., who reported the prevalence of ribosomal mutations to be 6.8% among macrolide-resistant pneumococci in Belgium (107). In Canada ~ 4% of erythromycin-resistant pneumococci harboured a ribosomal mutation (358) and 6.5% in the USA (79), although in a later study the respective figure was 1.6% for the USA (107). Globally, the prevalence of ribosomal mutations was 1.9% in 2003-2004, ranging among countries from 0-18% (107). (Farrell et al., Int J AA, 2008). This is a slightly higher rate than reported in 1999-2000 (1.5%) by the same authors (108). Ribosomal mutations can also exist simultaneously with other resistance genes (288), as was illustrated in our study.

Of all observed ribosomal mutations, A2059G seems to be the most common mutation converting macrolide resistance in pneumococci that are negative for common macrolide resistance determinant (92, 108, 288, 358). This was also the case in our study, although A2059C was previously more frequent in Finland (273, 275). Six novel mutations which, to our best knowledge, had not previously been described in pneumococci were found in this study. Two strains had a mutation in the highly conserved region $_{63}$LPWRQKGTGRAR$_{74}$ of the L4 protein. One of these had a glutamic acid (E) insertion after position 68, and the other strain had a glycine-glutamine-lycine (GQK) insertion after the same position. Earlier studies have suggested that mutations in this area are linked to macrolide resistance in pneumococcus (108, 275, 332). The role of the other four novel mutations, i.e. substitutions $T_{94}I$ and $V_{205}G$ in L4 or $R_{22}C$ and $A_{101}P$ in L22, remained unresolved. In particular, threonine replacement with isoleucine at position 94 in L4 could be significant, since threonine contains a reactive hydroxyl group, which is absent in the isoleucine molecule.

5.2 Telithromycin

Heterogeneous telithromycin resistance in pneumococci

The main observation of this thesis was the presence of heterogeneous telithromycin resistance among pneumococci. In the first pneumococcal collection (MILL-TELI02), 26 pneumococcal isolates carrying *erm*(B)

expressed telithromycin resistance that was manifested by the presence of bacterial colonies inside the inhibition zone around the telithromycin disk. Heterogeneous telithromycin resistance was also detected in nine *erm*(B) isolates of the invasive pneumococcal collection in 2002-2006. This type of telithromycin resistance has not previously been described. The majority of pneumococcal isolates derived from the colonies growing inside the inhibition zone of telithromycin disks (i.e. zone isolates) showed high-level resistance to telithromycin in agar diffusion performed in a CO_2-rich atmosphere. It was also evident that telithromycin resistance of the zone isolates was stable, homogeneous (consistent and clear growth pattern near telithromycin disk) and highly expressed (narrow inhibition zones and high MICs). Therefore, it can be concluded that some pneumococcal strains carrying *erm*(B) harbour a minor population of bacterial cells capable of expressing telithromycin resistance *in vitro,* which, according to telithromycin MICs, may be clinically significant. At least this characteristic can be considered to offer an advantage in the presence of antimicrobial pressure favouring the selection of resistant isolates. Previous laboratory experiments have shown that *erm*(B)-positive pneumococci require only two to three passages in telithromycin in order to achieve telithromycin resistance that is stable and maintained without continuing selective pressure from the antimicrobial agent (80, 349).

The results of the agar dilution method used in this study were in accordance with the results of disk diffusion testing: the vast majority of isolates showing heterogeneous telihtromycin resistance in disk diffusion testing had a TEL MIC $\geq$ 2 mg/L according to the agar dilution method. It should be noted that telithromycin MIC $\geq$ 2 mg/L is a non-susceptibility breakpoint set by the CLSI for the broth dilution method performed in normal atmosphere due to which conclusions need to be drawn cautiously. When testing with the CLSI broth microdilution method, however, the majority of pneumococcal isolates showing heterogeneous resistance to telithromycin were classified as telithromycin susceptible. Therefore, this method might be considered as deficient in detecting this type of resistance in pneumococci. Furthermore, these results might indicate that the expression of telithromycin resistance is more efficient in the presence of CO_2, as was considered by some authors to be the case with erythromycin and clindamycin (117). To conclude, we suggest that pneumococcal strains showing the MLS_B phenotype, or known to be positive for *erm*(B), should not be considered as susceptible to telithromycin unless susceptibility testing, preferably with disk diffusion

method, is performed. It should also be further discussed and investigated whether the susceptibility testing of pneumococci in ambient air with existing breakpoints underestimates the occurrence of telithromycin resistance.

The occurrence of telithromycin resistance

Apart from 26 heteroresistant pneumococci, two *mef*-positive isolates were classified as non-susceptible to telithromycin due to the reduced diameter of the inhibition zone. If this result was extrapolated to the population of 1007 pneumococci from which these isolates were derived, 2.8% of pneumococcal isolates in Finland were non-susceptible to telithromycin in 2002, the year in which telithromycin was launched on the market in Finland. Several studies in numerous countries have suggested the prevalence of telithromycin resistance to be less than 1% (110, 120, 229). One exceptional observation was from Taiwan, where Hsueh and colleagues reported that 16% of 936 pneumococcal isolates had a telithromycin MIC ≥ 1 mg/L in 2000-2001 (171). The majority of pneumococci with reduced telithromycin susceptibility in that study had an erythromycin MIC > 256 mg/L, which refers to the presence of *erm*(B) in the genome of these strains. Taiwan is a country with a very high rate of erythromycin resistance, and up to 94% of pneumococci are resistant to this agent (171). However, the methodology used in the Taiwanese study has subsequently been criticised (109). As mentioned previously, the PROTEKT study documented the worldwide prevalence of telithromycin resistance in pneumococci as being ≤ 0.3%, with no upward trend observed (120). A low prevalence of telithromycin resistance was also observed in USA: in 2001-2004, 0.01-0.04% of tested pneumococci were non-susceptible to telithromycin (187), while in 2005-2006 the respective proportion was 0.6% (74). In Canada similar prevalence was reported for 2002 (283).

Nevertheless, there are some signs of the emergence of telithromycin resistance, although so far the evidence has been restricted to individual case reports. To our best knowledge, the first clinical pneumococcal isolate resistant to telithromycin was described by Tait-Kamradt and co-workers in 2001. The isolate had been derived from conjunctival discharge in a 1-year-old boy in Canada as early as in 1996 (334). The first macrolide and fluoroquinolone treatment failure from which telithromycin-resistant pneumococcus was isolated was published in 2003 (271). According to this study, the development of telithromycin resistance (MIC 16 mg/L) during

clarithromycin and ciprofloxacin therapy appeared in a 71-year-old man in Spain in 2002. The cultured pneumococcal strain had an A2058G mutation in domain V of 23S rRNA in addition to a six amino acid (RTAHIT) insertion between amino acids T_{108} and V_{109} in the L22 protein. The isolate had ST180 and possessed serotype 3. The patient was cured with vancomycin therapy (271). Two years later, two reports of telithromycin treatment failures were published (106, 135). In one of these reports the patient was an 87-year-old woman with pneumococcal pneumonia who had chronic obstructive pulmonary disease as an underlying disorder together with a history of several treatments with macrolides. Telithromycin therapy was started but the patient's condition worsened. Bacteriological culture yielded a pneumococcus expressing TEL MICs of 1 mg/L in ambient air, but, interestingly, 8 mg/L when incubated in a CO_2-enriched atmosphere. The isolate had reduced susceptibility to penicillin in addition to resistance to macrolides, lincosamides, trimethoprim-sulfonamides and tetracycline. The patient recovered after telithromycin was replaced with amoxicillin (135). The other report described a treatment failure and the development of telithromycin resistance during telithromycin therapy in a 29-year-old woman who had acute exacerbation of chronic bronchitis (106). Azithromycin therapy was initiated, but was switched a few days later to telithromycin based on susceptibility testing. In the initial testing the pneumococcal isolate showed resistance to fluoroquinolones, penicillin and an M phenotype to macrolides (ERY MIC = 16 mg/L and AZM MIC = 32 mg/L), but was susceptible to telithromycin, showing MICs of 0.12 mg/L and 0.25 mg/L, respectively, in a normal atmosphere and in CO_2. The patient intially responded well to telithromycin therapy but her condition later worsened. This time the telithromycin MICs of the bacterial isolate were 256 mg/L and 512 mg/L by the agar dilution and broth dilution method, respectively, in a normal atmosphere, but in CO_2 it was 1024 mg/L. The later isolate had a constitutive MLS_B phenotype, although both the previous and the later isolate carried the *mef*(E) gene. Both isolates also had an $S_{20}N$ substitution in L4 compared to a wild type R6 *S. pneumoniae* strain. Further studies revealed, however, that the later isolate had an A2058T mutation in domain V of 23S rRNA and a three amino acid $_{98}PIN_{102}$ deletion in the L22 protein, which were missing in the previous isolate. Both the previous and the later isolates were identical in PFGE analysis and had the same mutations in *gyr*A, *gyr*B, *par*C and *par*E genes conferring fluoroquinolone resistance (106). Laboratory observations have provided evidence that telithromycin is only bacteriostatic against pneumococci with *erm*(B) (4, 202) and that

telithromycin concentration in lung tissue is not sufficient to kill pneumococci with an elevated MIC (2-8 mg/L) to telithromycin (4). Although telithromycin treatment failures still seem to be rare, the use of telithromycin in treating pneumococcal infections caused by a macrolide-resistant isolate should be carefully considered and at least susceptibility testing should be performed prior to treatment. In the case of an unfavourable response to telithromycin therapy, new specimens should be taken to rule out the presence of telithromycin-resistant pneumococci, because isolates with a macrolide resistance mechanism are prone to develop high-level resistance during treatment.

Telithromycin resistance mechanisms

According to laboratory experiments, telithromycin MICs of A2059G, C2610U or C2611U laboratory-derived mutants did not differ from wild type isolates, whilst strains having an A2058U mutation had a slightly elevated telithromycin MIC (0.25-1 mg/L) and a mutant with an A752 deletion had a telithromycin MIC of 4 mg/L (52). Apart from interaction at position A2058 in domain V, telithromycin interacts at nucleotide A752 in domain II of 23S rRNA, which improves the drug's affinity to resistant ribosomes (1). Therefore, changes at this position can reduce the susceptibility to telithromycin. Laboratory experiments have shown that a high telithromycin MIC in pneumococcus can be obtained after serial passage of an *erm*(B)-positive isolate to telithromycin (349). In one of such isolates with a telithromycin MIC > 32 mg/L a 210 bp deletion was observed in the upstream region of *erm*(B) together with an L94Q change in the L22 protein. Interestingly, the same study described telithromycin-resistant pneumococci in which no deletions in the regulatory sequence of *erm*(B) or ribosomal mutations could be detected (349).

In clinical isolates, telithromycin MICs of 1-16 mg/L have been detected in pneumococcal isolates having a $_{71}$REKGTG$_{72}$ insertion in the L4 protein (3.12 mg/L), a $_{108}$RTAHIT$_{109}$ insertion in the L22 protein (1 mg/L), a K$_{68}$Q mutation in the L22 protein (1 mg/L), an A2058G mutation together with a $_{108}$RTAHIT$_{109}$ insertion in the L22 protein (8-16 mg/L) and a $_{92}$VRPR$_{93}$ insertion in the L22 protein (2 mg/L) (see Table 1). Slightly increased telithromycin MICs have also been reported in association with other mutations. For example, in a Finnish study, four isolates with an A2059C mutation had a telithromycin MIC of 0.125 mg/L, but isolates with an

A2059G or mutation alone or combined with a $_{69}GTG_{71}$ to TPS mutation had a telithromycin MIC of 0.25 mg/L and 0.5 mg/L, respectively (275). It seems, however, that higher telithromycin resistance is especially associated with the presence of *erm*(B) in pneumococci and deletions detected in the *erm*(B) leader sequence or the SD2 site of *erm*(B) (166, 360). Such changes can also occur with a combination of a $_{69}GTG_{71}$ to TPS mutation in ribosomal protein L4 (360, 362). It is known that ketolide resistance in *Streptococcus pyogenes* correlates with the degree of dimethylation by Erm and that a strain with a deletion in the *erm*(B) leader sequence leads to high degree of dimethylation (95). Recently, this was also shown to be a case in *S. pneumoniae* (360). In our study, both parent and zone isolates had identical *erm*(B), 23S rRNA, L22 and L4 sequences. We could not find deletions in the *erm*(B) leader sequence or mutations known to confer macrolide or ketolide resistance in these isolates (paper III).

Clonal relationship of the telithromycin resistant isolates

Our results demonstrated that the capability of expressing heterogeneous resistance can be detected in several pneumococcal strains. Altogether, seven distinct sequence types that had this capacity were identified. The most common telithromycin resistant strain in our study, ST193, is a 19A serotype variant of the PMEN clone Greece^{21}-30. This clone has earlier been described in Greece, Brazil, the United Kingdom, Vietnam and Italy (133, 329). Apart from Finland, serotype variant 19A of this clone has been observed in Italy. Otherwise, the serotypes of the members of this clone have been reported to possess serotypes 21, 18C, 14, or are non-typeable (www.mlst.net). ST133 has previously been reported in Spain with the same serotype as our two strains, 18C (www.mlst.net). Two other strains described in our study were representatives of well-characterised PMEN clones. One of these had ST273, which is a representative of the penicillin-susceptible but erythromycin-resistant PMEN global clone Greece^{6B}-22. Apart from Greece, this clone has been detected in Iceland, Israel, Portugal, Italy, Germany and Switzerland (www.mlst.net). The other strain having ST271 was a single locus variant of a multi-drug-resistant Taiwanese^{19F} clone, the sequence type of which is 236 (45). Our isolate carried both *erm*(B) and *mef*(E) macrolide resistance determinants. The same variant has spread worldwide: according to an international study, over 85% of pneumococcal isolates having the double mechanism belonged to one major clonal complex representing ST271 (115). Our second isolate with the double mechanism had a novel sequence type,

ST2248. Two other novel sequence types, ST2306 and 2307 with serotypes 14 and 19F, respectively, were also observed.

5.3 Resistance to other antimicrobials

Penicillin

A continuous increase in penicillin non-susceptibility from 8 to 16% was observed among invasive pneumococci during the four-year study period. Compared to 1999-2000, a four-fold increase was observed in penicillin non-susceptibility among invasive pneumococci in Finland (273). As in the case of erythromycin resistance, the highest penicillin non-susceptibility rates were detected in children. Regarding penicillin non-susceptibility, the European map is very similar to that for erythromycin resistance, with south-western Europe and Mediterranean regions being the resistance hot-spots, although penicillin non-susceptibility prevalences are to some extent lower than those for erythromycin (293). France and Spain are at top of the list of European countries with the highest penicillin non-susceptibility (84, 258, 287, 293). In Spain, however, a decline in overall penicillin non-susceptibility from 39.5 to 33% was observed during 2001-2003, the decline being largest in children under 14 years old, from 60.4 to 41.2% (258). The authors suggested that increased vaccination of children with the PCV-7 as well as reduced antimicrobial consumption in the community could be the possible explanatory factors for this trend (258). In Portugal, the proportion of pneumococci intermediately susceptible to penicillin increased from 12 to 23% in 1994-2004 with a simultaneous decrease (from 4 to 0.9%) in the proportion of isolates showing high level penicillin resistance (90). A study from the USA also reported a change towards intermediate resistance: in 2000-2004 the proportion of intermediately resistant isolates increased from 12.5% to 20% while the proportion of resistant isolates decreased from 26.3% to 16.5% (187).

Regardless of the overall high penicillin non-susceptibility percentages in many European countries, some countries such as Denmark, Sweden, Norway, Iceland, the Netherlands and the UK have so far managed to keep penicillin non-susceptibility prevalences between 0-10% (46, 293). In Canada, 15% of pneumococci were penicillin non-susceptible in 2002 and approximately half of these isolates expressed a high level of penicillin

resistance (283). In the USA, on the other hand, the proportion of penicillin non-susceptible isolates exceeded 37% in 2005-2006, and 16% of all pneumococci are fully resistant to penicillin (74). Overall, on the global level, penicillin non-susceptibility remained at 37% in 2001-2004, with wide variation among countries (120). The worldwide prevalence of pneumococci fully resistant to penicillin (~24%) exceeds that of intermediate isolates (~14%) (120). In this context, the situation in Finland is good, since only 4% percent of pneumococci are fully resistant to penicillin.

The penicillin-resistant invasive pneumococcal population in Finland seems to be genetically rather heterogeneous. Several international PMEN clones with their variants with different type of macrolide rsistance genes, as well as strains with new sequence types were detected, indicating the continuous exchange of genetic material between the pneumococcal isolates as well as the liability and competence of these isolates. The factors affecting clonal survival are complex and not fully understood. They can include antimicrobial use, the development of herd immunity (i.e. use of vaccines), and characteristics of the strain itself (14, 315, 316), including virulence factors. The most frequent clone detected in Finland, Spain9V-3 or ST156, has been observed to be one of the most successful global clones (133, 150, 306, 315, 366). This clone was recently found to be associated with increasing penicillin resistance in Sweden (315). The Swedish study group also proved that members of this clone carried a *rlrA* pilus islet that was shown to make the clone more successful in an animal model of carriage compared to the strains that lack this gene (315). The pilus gene was also found in ST156 isolates of our study.

Ceftriaxone

Penicillin non-susceptible pneumococci are often still susceptible to second or third generation cephalosporins, although MICs are higher compared to penicillin susceptible strains (38, 265). However, the susceptibility of penicillin-resistant pneumococci to cephalosporins is difficult to predict and should therefore always be tested. It is also possible for an isolate to have a high MIC to cephalosporins and just a slightly elevated MIC to penicillin. This type of isolate was already described at the beginning of the 1990s (124). It seems that ceftriaxone-resistant strains are extremely rare in Finland, possibly due to the relatively low proportion of pneumococci that are highly resistant to penicillin. However, reduced susceptibility was detected in 3-4%

of isolates, which warrants careful surveillance in the future. Ceftriaxone non-susceptibility is more frequent in countries with a high prevalence of penicillin resistance. In South Korea and Singapore > 15% of pneumococci were non-susceptible to ceftriaxone in the late 1990s and early 2000s (62, 208). In Taiwan, the prevalence of ceftriaxone non-susceptibilty increased from 3-4% (before 2004) to 7.4% (by 2005) (60). In North-America in 1998-2004, 6.3% of pneumococci were non-susceptible to ceftriaxone, but the MIC_{90} was low, 1 mg/L (195). In Brazil, high level ceftriaxone resistance (MIC $\geq$ 4) was also low (0.5%) for the same period (53). In Portugal, ceftriaxone non-susceptibility was observed to decrease from 4% to < 1% in 1994-2006 when a shift from penicillin resistance to penicillin intermediate susceptibility was observed (90). In the Barcelona area of Spain, non-susceptibility to ceftriaxone was recently reported as 9% (343). In Italy, nearly all pneumococci (99.9%) were susceptible to ceftriaxone in the period from 1993-2004 (244). In Hungary, a slightly lower susceptibility rate (99%) was reported in 1998-2004 (145). In Canada, only a few isolates were non-susceptible to ceftriaxone in 2002 (283). This was in contrast to another Canadian study that documented 5% ceftriaxone non-suceptibility among pediatric isolates, the majority of which were fully resistant to penicillin (27). In Greece, 1% of pneumococci recovered from carriers under 6 years old were non-susceptible to ceftriaxone, while the respective figure for adult clinical isolates was 5.6%. The proportions of isolates showing full resistance to ceftriaxone were 0.1 and 2.2%, respectively (282). It must be noted however, that the comparison between the studies is sometimes difficult because of differences in representativeness of the investigated isolates, used breakpoints and other methods.

Fluoroquinolones

Fluoroquinolone resistance is rare in Finland ($\leq$ 0.5%) and no increasing trend was observed. In Europen countries, 1-2% of pneumococci were reported to be resistant to fluoroquinolones in 2004-2005 (90 , 91, 287). It has also been speculated that fluoroquinolone resistance would emerge rather slowly due to the possible fitness cost of the resistance to bacterial cells (300). However, in Italy, up to 15% of pneumococci were resistant to ciprofloxacin and 5.6% to levofloxacin in a multicentre study that investigated bacterial strains collected in 2001-2004 (87). In Canada an

increasing trend in ciprofloxacin resistance from < 1% in 1997 to 4.2% in 2005 was also observed (Adam et al., AAC, 2007, 51, 198-207). A similar observation was reported in Portugal: from 0.5% ciprofloxacin resistance in 2002 to 3.5% in 2004 (90).

Multiresistance

In our study over 70% of PNSP isolates of this study were also non-susceptible to erythromycin. It seems that this proportion has increased to some extent compared to year 2000 when 62% of PNSP isolates were erythromycin non-susceptible (274). However, due to relatively low proportion of resistance to other antimicrobials, multiresistance situation is still satisfactory in Finland, particularly what comes to invasice isolates. For comparison, In Portugal the prevalence of multiresistace in invasive pneumococci increased from 4% to 15.6% in 1994-2004, paraller with the increase of tetracycline and erythromycin resistance (90). In general, the higher resistance to erythromycin or penicillin in pneumococci, the higher prevalence of multiresistance is detected. For example in USA multiresistance percentages ranges from 9 to 25% by region with the highest rates detected in those areas with high prevalence of penicillin and erythromycin resistant strains(74, 187).

5.4 Spread and control of resistance

The clonal spread of resistant strains seems to be efficient in pneumococci (12, 83, 250). In Finland the majority of penicillin-resistant pneumococci also belong to well-known global clones, although the number of clones is quite high. In addition, new variants and novel sequence types indicate the continous exchange of genetic material between pneumococci. This is also reflected by the presence of different types of resistance genes in strains of the same sequence type.

Apart from clonal spread, many studies have shown that the higher the antimicrobial pressure, the greater the resistance in pneumococci (25, 35, 89, 200, 293). This was also clearly illustrated in a study including pneumococcal resistance data and antimicrobial consumption data from 15 European countries (293). Compared to other European countries, Finland had a medium level of antimicrobial consumption (~ 17 DDD per 1000 inhabitants), the range being 9.7-31.6 DDDs in 1998-2004. Antimicrobial use was highest in France and lowest in the Netherlands (293). As earlier reviewed, these countries have the highest and the lowest antimicrobial prevalence, respectively. The Nordic countries represented in this study had a lower overall consumption of antimicrobials than Finland. In the light of this information, it is not surprising that Finland is a leading country in Northern Europe in terms of resistance rates.

However, even though it is widely accepted that resistance and consumption are linked, the magnitude of the relationship is not always clear. A reduction in antimicrobial consumption does not always result in a reduction in resistance. For example, due to the worrying evolution of resistance in the 1990s, public health authorities in the UK and Ireland launched campaigns to raise awareness of antimicrobial resistance (221). As a consequence, the total sales per capita of erythromycin and betalactams decreased significantly from 1994-2004 in the UK, but not in Ireland. This was followed by decrease in penicillin non-susceptibility among pneumococci in both countries, while macrolide resistance remained rather stable (221). In Canada, the emergence of high-level penicillin resistance and multiresistance was observed in 1997-2002, despite a decrease in antimicrobial consumption (369). In Iceland, meanwhile, an increase in PNSP in healthy carriers was observed in some regions, although the consumption of antimicrobials decreased after active public health intervention (12). In Norway, on the other hand, macrolide resistance increased despite low macrolide consumption in 2001-2005 (319). These examples are interesting and raise questions such as the following: 1) Why is a decrease in resistance associated with reduced consumption for one antimicrobial (betalactam consumption decrease – penicillin non-susceptibility decrease), but not necessarily for another (erythromycin consumption decrease – erythromycin resistance stable)? 2) Why does the resistance to one antimicrobial decrease but that to another remain stable, even though their consumption does not change? 3) How large a decrease in consumption and how long a duration of action is needed before the effect on resistance rates can be seen for each antimicrobial? 4) What is the effect of

the starting level of resistance (low vs. medium vs. high) on resistance trends when a follow-up period is started? Finally, 5) How long a follow-up period is needed before any conclusions about trends can be made (many studies calculate statistical parameters for a short follow-up period)?

It has been suggested that to reduce the antimicrobial prevalence in pneumococcus, a large reduction in antimicrobial consumption is needed on the population level (237). Apparently, many other factors also affect resistant rates such as population density, age, geographical location, isolate source (345), herd immunity, pneumococcal population dynamics, the clonal spread of resistant isolates (12, 319) and socioeconomic factors (131). Furthermore, the importance of taking pharmacokinetic and pharmacodynamic parameters into account to confirm the bacteriological cure and to avoid the selection of resistant isolates when treating infections in individual patients should not been underestimated. To summarise, in controlling antimicrobial resistance, a multidisciplinary approach is therefore needed that includes continous surveillance, education and feedback, the use of vaccines and the development of new drugs.

5.5 Resistance in relation to conjugate vaccine serotypes

Regardless of the fact that there has been considerable surveillance and research activity over the years, and regardless of the many specialists and committees expressing their concerns, respective actions and interventions to control the resistance problem have, in general, been too deficient to have an effect. However, the 7-valent vaccine has had a dramatic effect in reducing the incidence of invasive pneumococcal disease, not only in children but also in other age groups, due to heard-immunity (280). In some places, a decrease in resistance has also occurred after launching the vaccine. According to a recent evaluation, a group of experts decided to recommend the introduction of this vaccine in the Finnish national vaccination schedule (KTL 2009, http://www.ktl.fi/portal/2920). Our results showed that the approximate hypothetical serotype coverage by PCV-7 was 58%, ranging from 54% (2003) to 62% (2006). This result is in accordance with many others (236, 282). In Finland the conjugate vaccine would cover 80% of the erythromycin resistant and 86% of the penicillin non-susceptible isolates. The serotypes most frequently non-susceptible to erythromycin or penicillin in Finland were

14, 9V, 19A, 6B and 19F, as in many other countries (20, 50, 83, 121, 220, 350). Serotype 23F was the third most common serotype among invasive isolates, but the proportion of resistant isolates in this serotype was relatively low compared to other reports. Serotype 23F has been shown to be frequent among resistant isolates elsewhere (50, 227, 278).

Regardless of the conjugate vaccine's outstanding positive effect on the incidence of invasive pneumococcal disease, there are some concerns. Several studies have documented that the invasive pneumococcal disease caused by either non-vaccine serotypes (particularly serotypes 35B, 15C, 15B, and 33F) or vaccine-related serotypes (particulary 19A) has increased in the post-vaccine era (136). The reduction of pneumococci of vaccine serotypes forms a free ecological niche to be filled by non-vaccine and vaccine-related pneumococci. This can occur by 1) the expansion of clones of non-vaccine/vaccine-related serotypes that were a minority in the pre-vaccine era (129), 2) the maintenance/emergence of existing clones that switch their vaccine serotype capsule to a non-vaccine or vaccine-related serotype by capsular switch (=same sequence types remain, only the capsular type they posess changes) (261) or 3) by the recombination of genetic material and the generation of new clones (148).

Because of the high prevalence of antimicrobial resistance in most common pneumococcal serotypes, one could expect the introduction of the PCV-7 into the national vaccination programme would reduce the proportion of antimicrobial resistance in Finland. Follow-up studies performed in the post-vaccination era suggest, however, that the longer term impact of the vaccine on the epidemiology of antimicrobial resistance is not yet clear. Some investigators have observed a reduction in antimicrobial resistance (32, 230, 335), while others have not (89, 129, 236, 238). Current evidence suggestes that the prevalence of antimicrobial resistance can rapidly increase in non-vaccine/vaccine related pneumococci (112, 172). The data also indicate that the PCV-7 does not reduce the overall pneumococcal carrier rate in children or antimicrobial resistance among these isolates (172, 216, 268). This is probably because the vaccine serotypes are replaced by non-vaccine or vaccine-related serotypes due to capsular switching, while the genotype remains same (136, 148, 249, 314, 322). The introduction of the pneumococcal vaccine into the national vaccination schedule in Finland will certainly affect pneumococcal epidemiology. The future will reveal whether this will have an impact on antimicrobial resistance. Emerging macrolide and

penicillin resistance in pneumococci is a public health concern and may create problems in the treatment of pneumococcal infections. Therefore close surveillance of antimicrobial resistance and of changes in the pneumococcal population is of utmost importance in the near future.

6. Conclusions

1. There is a high prevalence of erythromycin resistance in Finland since nearly 30% of all pneumococci and 46% of pneumococci of small children are non-susceptible to erythromycin. Therefore macrolides can not be recommended for the treatment of pneumococcal infections without susceptibility testing to avoid possible treatment failures.

2. The main observation of this thesis was the presence of heterogeneous telithromycin resistance among pneumococci carrying *erm*(B). Such isolates harbour a minor population of bacterial cells capable of expressing high level telithromycin resistance *in vitro,* which may be clinically significant. However, CLSI broth microdilution method does not favour the detection of pneumococci with this resistance type. Therefore disk diffusion susceptibility testing of *erm*(B) positive pneumococci or pneumococci showing MLSB phenotype is recommended.

3. The presence of more than one clone among telithromycin resistant pneumococci suggests that the capability to express telithromycin resistance have developed independently in separate ancestors. The results also indicate that this type of resistance have capability to spread, since the majority of such isolates belonged to global clones.

4. Prior its widespread use, the proportion of telithromycin resistant isolates was rather high (2.6%) in Finland if compared to other studies. Partly this can be explained by the use of different methodology, but because the similar result was achieved with two different methods in this study, we consider our result as reliable. Close monitoring of telithromycin resistance is needed in the future.

5. Apart from macrolide resistance, also proportion of penicillin non-susceptible isolates is emerging, being already 16% in 2006. However, the prevalence of isolates fully resistant to penicillin is still low. The results showed that there are several international PMEN clones and their new variants indicating which continuous exchange of genetic material between the strains. Our results provide baseline information on pneumococcal epidemiology of the prevaccine era and allow the follow-up changes which occur in pneumococcal population after the PCV-7 vaccine is introduced.

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